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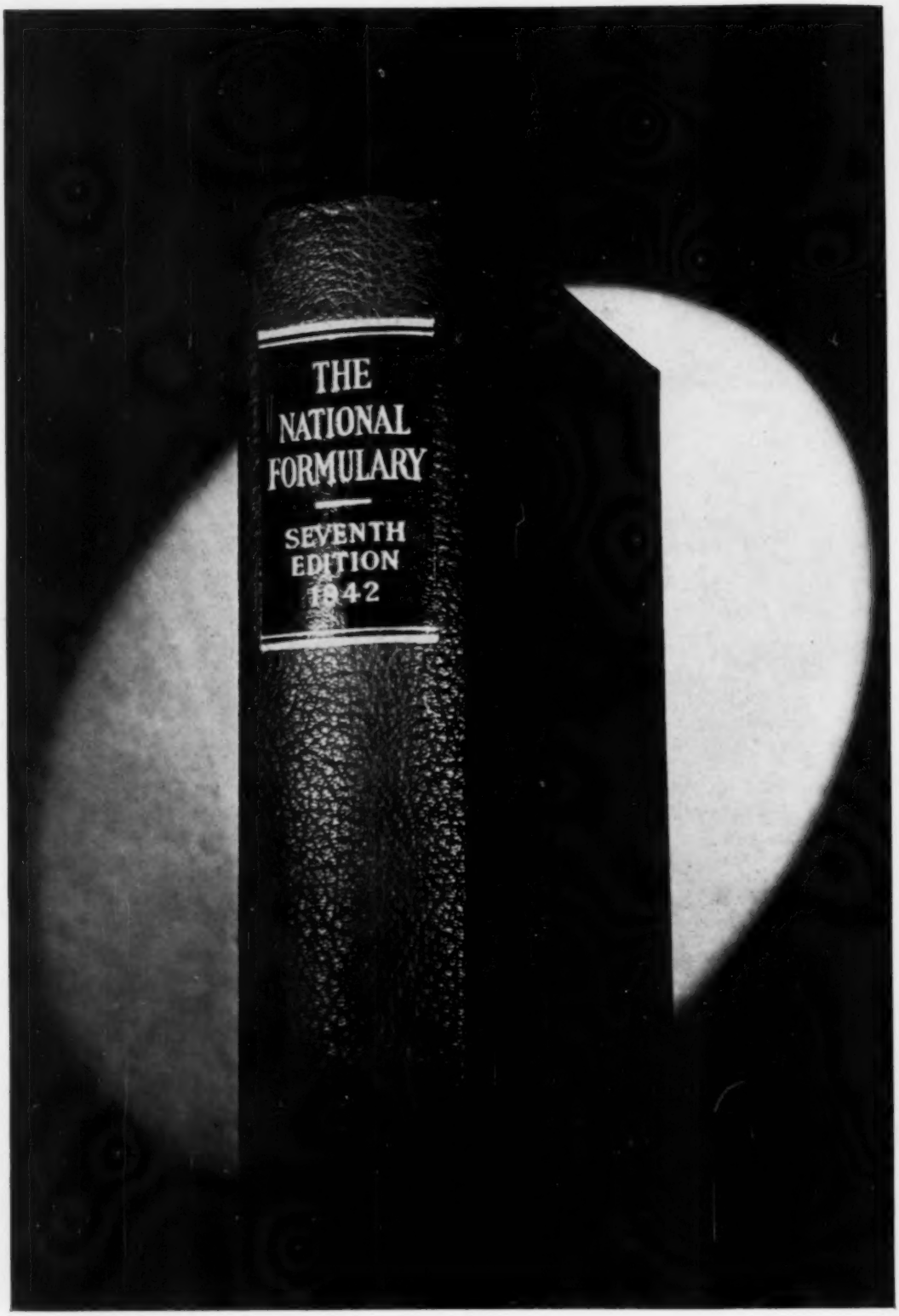
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JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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The Assay of Digitalis

III. The Potency of U. S. P. XII Digitalis*†

Herbert A. Braun and Lloyd C. Miller

The question of the relative potencies of U. S. P. X and U. S. P. XI Digitalis has been reviewed by Edmunds (2), who has also set forth the events leading up to the adoption of the International Standard Digitalis (1926) and the U. S. P. Reference Digitalis Powder (3). When the latter reference standard became available, it was generally expected that its use would result in fixing the U. S. P. XI standard of potency at a level about 25% above the U. S. P. X potency. Considerable literature developed which raised doubt as to whether this expectation was actually realized. Some observers have reported that the increase was of the order of 50% to 70% (4, 5, 6, 7, 8). The exhaustion of the original (1926) supply of international standard as well as the acknowledged unsuitability of ouabain as a reference standard for digitalis are but two factors of many which preclude the satisfactory resolution of the controversy.

Difficulties of a similar nature complicate the problem of estimating the expected decrease in potency which will result in changing from U. S. P. XI to U. S. P. XII standards of strength. The object of this report is to make available certain data bearing on this question. These data were obtained in comparisons of the various reference digitalis standards among themselves and in assays of samples of commercial digitalis preparations which have entered interstate commerce. The data justify the conclusion that the potency of U. S. P. XII Digitalis will be substantially less than that of U. S. P. XI Digitalis and indicate the extent of the decrease attributable to the change in reference standard and, in general, the decrease arising from the change in test animal.

Most of the material used in this study represents official samples submitted to this laboratory in the course of regulatory activities connected with the enforcement of the Federal Food, Drug, and Cosmetic Act. The series does not represent a cross section of the digitalis in interstate commerce, since an inordinate proportion of the samples reported have formed the basis for regulatory action which has terminated. Such samples are, as a matter of course, much

* Received July 3, 1942, from the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

† The second paper of this series, entitled "The Assay of Digitalis. II. Absorption as Influenced by the Site of Injection" (1), was presented before the American Society for Pharmacology and Experimental Therapeutics at its annual meeting in New Orleans, March 13–16, 1940.

more exhaustively examined than those which give evidence of meeting the official requirements. The additional examination has included assays on cats, the results of which permit a calculation of the potency in terms of the new U. S. P. XII requirements. In view of the great interest in the changing standards of strength, it seems desirable to publish the information so obtained and to support it by similar data on other samples whose potencies are within the allowed tolerances. The data are further supplemented by extensive comparisons of the U. S. P. Reference Digitalis Powder with both of the International Standard Digitalis powders (1926 and 1936) and the "study powder."[1]

EXPERIMENTAL

Preparatory to assay of those samples in solid form (powders and tablets), tinctures were prepared by adding to a weighed amount of the preparation in a glass stoppered centrifuge tube (9), a volume corresponding to 10 cc./Gm. of U. S. P. digitalis menstruum. In the case of powdered extracts, a liquid preparation was obtained similarly by using a proportion of 10 cc. of menstruum per 0.25 Gm. as directed in N. F. VI. The mixtures were shaken mechanically and continuously for 24 ± 2 hrs. Following this maceration period the liquid phase was clarified by centrifuging and decanted into a hard glass bottle in which it was stored under refrigeration. Suitable precautions were taken to prevent evaporation during storage. The tinctures were usually assayed promptly and in no case longer than thirty days after their preparation.

In carrying out the U. S. P. XI method, all the specified directions were followed closely. Test dilutions of the standard and of the two or three preparations to be assayed were prepared fresh each day and were so adjusted that the dosage per Gm. of frog was contained in 0.02 cc. Each test dilution contained approximately 23% of alcohol regardless of the actual concentration of digitalis which varied, of course, with the dosage. In routine assays, three dosage levels were used which stood in relation to each other as $1 : \sqrt{2} : 2$ so that the logarithm of the dosage interval was 0.15. When the low dose of such a series produced positive results (systolic standstill of the ventricle) in 25% of the frogs injected, such a dosage range usually assured approximately 50% and 75% of positive results with the intermediate and high doses, respectively. Every effort was made to insure homogeneity within the various groups of frogs receiving the different dosage levels. This precaution not only complies with the principles of good practice, but also makes possible the use of recently developed statistical methods (10) in calculating the most probable potency and its standard error, which is a measure of the reliability associated with the estimate of potency.

In using the 18-hr. (overnight) method, the test dilutions were prepared as for the U. S. P. XI method except that the three doses were usually related as $1 : \sqrt[4]{2} : \sqrt{2}$ so that the dosage interval corresponded to log-dose 0.075. As with the U. S. P. XI assay, precautions were observed for insuring homogeneity among the frogs so that the same means of calculating the potency and its standard error were available.

In employing the U. S. P. XII method in which the cat is the test animal, the directions for selecting and preparing the cats and for preparing and injecting the test dilutions were followed.[2] Ordinarily six cats were injected simultaneously, two being injected with the diluted Standard Preparation of Digitalis, and two each with dilutions of two different preparations to be assayed. In this way, day-to-day (secular) variations in the responses of the cats were minimized. It was observed that such variations could assume considerable magnitude over and above what may prove to be a seasonal variation in the average lethal dose of the standard preparation. In this study the most probable potency and its standard error were determined by a method of calculation[3] essentially that suggested by

[1] Grateful acknowledgment is hereby recorded for adequate supplies of International Standard Digitalis (1936) and of the study powder obtained through the kindness of Professor E. Fullerton Cook, General Chairman of the U. S. P. Committee of Revision.

[2] The U. S. P. XII assay directions substantially in their final form were made available through Dr. E. E. Nelson, Chairman of Subcommittee No. 3 on Biological Assays of the U. S. P. Revision Committee.

[3] The calculation is a slight modification, with certain corrections, of that developed by Dr. Bliss for consideration by the subgroup on digitalis assay of the U. S. P. Subcommittee No. 3 on Biological Assays. It is carried out as follows:

Express in logarithms the number of doses of test dilution required by each cat. To the logarithms of the observations on those cats receiving the standard, add the logarithm of the number of cc. of Standard Preparation of Digitalis contained in each 100 cc. of the test dilution of the standard. Average (arithmetically) the sums so obtained to get $\bar{y}_S$, the average log-dose of the standard. Similarly, add to each of the logarithms of the observations on the cats receiving the preparation being assayed (the unknown) the logarithm of the number of cc. of the unknown in each 100 cc. of the test dilution of the latter. Average these sums to obtain $\bar{y}_U$, the average log-dose of the unknown.

Obtain M, the logarithm of the number of U. S. P. Digitalis Units per cc. of the preparation being assayed, by subtracting $\bar{y}_U$ from $\bar{y}_S$.

Calculate the standard error of M, s_M, by one of the following formulas:

(*a*) Where an equal number of cats has been used on both standard and unknown,

$$s_M = \sqrt{\frac{S(y^2) - N(\bar{y}_S^2 - \bar{y}_U^2)}{N(N-1)}}$$

in which $S(y^2)$ = sum of the squares of the log-doses for all the cats used, N = number of cats on standard, $\bar{y}_S$ = average log-dose of standard and $\bar{y}_U$ = average log-dose of unknown. (*Continued, p. 227*).

TABLE I.—COMPARISON OF *USP* REFERENCE DIGITALIS POWDER WITH INTERNATIONAL STANDARD DIGITALIS POWDERS (1926) AND (1936)

Date	Method	Standard	No. of Animals	Amount of Standard Equivalent to 1 Gm. of *USP RDP* Grams	Log ± *S. E.*
May, 1936[a]	*USP* XI	*ISD* (1926)	179	2.06	0.3136 ± 0.033
June, 1936[a]			80	1.68	0.2253 ± 0.050
Dec., 1937[a]			130	1.93	0.2867 ± 0.039
Apr., 1939			180	2.24	0.3512 ± 0.0248
Weighted average, all assays				2.07 ± 0.08	0.3156 ± 0.0166
Mar., 1938	*USP* XI	*ISD* (1936)	330	1.37	0.1358 ± 0.0306
Apr., 1939			435	1.25	0.0982 ± 0.0237
Aug., 1939			540	1.31	0.1174 ± 0.0231
Sept., 1939			129	1.20	0.0809 ± 0.0646
Oct., 1939			380	1.25	0.0982 ± 0.0296
June, 1940			144	1.12	0.0504 ± 0.0345
Weighted average, all assays				1.27 ± 0.04	0.1028 ± 0.0121
Apr., 1939	4-Hr.	*ISD* (1936)	240	1.38 ± 0.10	0.1391 ± 0.0319
Mar., 1938	18-Hr.	*ISD* (1936)	380	1.29	0.1057 ± 0.0080
Aug., 1939			540	1.21	0.0811 ± 0.0166
Feb., 1940			160	1.37	0.1375 ± 0.0250
July, 1940			80	1.26	0.0991 ± 0.0258
Weighted average, 18-Hr. assays				1.27 ± 0.02	0.1036 ± 0.0067
June, 1938[b]	Cat	*ISD* (1936)	18	1.18	0.0711 ± 0.0351
Sept., 1941			12	1.11	0.0433 ± 0.0308
Nov., 1941			11	1.29	0.1100 ± 0.0336
Weighted average, 1941 assays				1.19 ± 0.06	0.0738 ± 0.0227

[a] These assays were carried out using the single-dose standard curve method which does not provide an inherent check on the accuracy and therefore the data do not yield an estimate of the standard error. The standard error values given are approximations obtained by taking into account the number of frogs used in these assays and the observed standard errors in three-dose assays.

[b] This assay on the cat involved a faster rate of injection than specified in the *USP* XII assay so that the mean death time was 39 min. It was not included in the weighted average.

Bliss and Hanson (11), in which the individual lethal doses are transformed to logarithms. In contrast to the method of calculation supplied in U. S. P. XII, this calculation facilitates combining the results of two or more assays in a weighted mean to which each individual result contributes in proportion to the information it affords. Assigning weights in this way involves the assumption that an assay yielding a low calculated standard error provides, in general, a more reliable estimate of the true potency and, therefore, more information relative to it than does a similar assay having a high standard error.

An effort was made to carry out sufficient assays by each method so that the calculated standard error of the weighted mean was not greater than 10% of the mean. Ordinarily to accomplish this, it was necessary to conduct at least three U. S. P. XI assays, each of which involved the use of 40 frogs on the standard and on each preparation being assayed, or a total of 160 for the assay of three samples. Two 18-hr. assays on the same number of frogs were quite sufficient because of the greater accuracy characteristic of this method, an observation first substantiated by objective evidence in the initial paper of this series (10). With the U. S. P. XII assay, a comparable accuracy usually results from employing six to nine cats on both the standard and the preparation to be assayed.

A Comparison of the U. S. P. Reference Digitalis Powder with the 1926 and 1936 International Standard Digitalis Powders.—During the period the U. S. P. Reference Digitalis Powder[4] has been available, numerous comparisons have been made in this laboratory to determine its relative potency in terms of the 1926 and the 1936 international standards. The results of all these assays are compiled in Table I, in which the individual assay values have been combined arbitrarily by months as a matter of simplifying the table. The data are expressed in terms of the amount of the respective international standard which is equivalent to 1 Gm. of *USP RDP* by the method of assay specified.

The exceptionally high potency of the *USP RDP*

[3] (*Continued*). (*b*) Where an unequal number of cats has been used on standard and unknown,

$$s_M = \sqrt{\frac{[S(y^2) - N_S\bar{y}_S^2 - N_U\bar{y}_U^2](N_S + N_U)}{(N_S + N_U - 2)N_SN_U}}$$

in which $S(y^2)$ = sum of the squares of the log-doses for all the cats used, N_S = number of cats on standard, $\bar{y}_S$ = average log-dose of standard, N_U = number of cats on unknown and $\bar{y}_U$ = average log-dose of unknown.

Where more than one assay has been made, combine the individual results by the procedure suggested by Miller, Bliss and Braun (10).

[4] Toward economy of space, the following abbreviations will hereafter be used: *USP* in referring to the U. S. Pharmacopœia, *USP RDP* in referring to the U. S. P. Reference Digitalis Powder; *ISD* (1926) and *ISD* (1936) in referring to the International Standard Digitalis (1926) and (1936), respectively, and *USP DRS* in referring to the U. S. P. Digitalis Reference Standard (1942).

is apparent at once from these data. By the *USP* XI method it appears to be more than twice as potent as the *ISD* (1926), the weighted average of all the assays indicating that 1 Gm. of *USP RDP* is equivalent to 2.07 Gm. of *ISD* (1926) or 207% of this international standard. The latter value is to be compared with the observation of Edmunds, Moyer and Shaw (3) that, in their hands, the *USP RDP* was 134.22% of *ISD* (1926). It was their value, of course, on which the "factor" for the *USP RDP* was based. The present observation of 207% is somewhat higher than any heretofore reported as may be seen in Table V. Because the supply of *ISD* (1926) was so severely limited, assays by methods other than the official were not attempted.

The comparisons of *USP RDP* and *ISD* (1936) bring out several points. With respect to methods, it is clear that there is no difference in the relative potencies of these two powders as indicated in results of frog assays involving three different periods of observation. While only two assays were carried out with the 4-hr. method, the results of these are not significantly different from the weighted mean results of either the *USP* XI or the 18-hr. assays. The latter represent a total of 14 and 9 assays respectively. The observation that the *USP* XI and the 18-hr. assays agree very well is in confirmation of the results obtained in the first comparison of the *USP* digitalis study using frogs (12). It is in conflict with the conclusion drawn by Edmunds, *et al.* (3), with respect to these two powders. As indicated below (Table V), their conclusion was that the *USP RDP* appeared stronger with the longer period of observation. It seems unlikely, in view of the limited number of frogs used in their assays, that this conclusion was justified. Differences of the magnitude they observed cannot be established with a satisfactory degree of certainty without the use of several hundred frogs.

Since the potency estimates between the three frog methods do not differ significantly, it is probably safe to combine them into a weighted mean. Doing this yields the observation that 1.0 Gm. of *USP RDP* is equivalent in the frog to 1.27 ± 0.02 Gm. of *ISD* (1936). By definition, 0.080 Gm. of the latter is established as currently representing the potency of 1.0 International Digitalis Unit; thus, 0.0630 Gm. of *USP RDP* exhibits in the frog an activity of 1 International Digitalis Unit. The difference between 0.0630 and 0.0745 Gm., the latter being the amount of *RDP* fixing the *USP* Digitalis Unit, is 15.4% and is a measure of the discrepancy between the *USP* Digitalis Unit and the International Digitalis Unit as both are currently defined under the provisions of *USP* XI. Actually, it is known that this must be an underestimate of the discrepancy since the factor of 0.8 for *ISD* (1936) is an average of results by three methods of assay. Actual assay on frogs (presumably by the overnight method) showed that *ISD* (1936) was 138% of *ISD* (1926) (13), so that, strictly speaking, a factor of 0.725 is more appropriate for relating the potency of the two standards by the frog method. On this basis the real difference between the *USP* and the international unit is closer to 23%. In any event, the discrepancy would scarcely seem to be of any great moment to the practicing physician and suggests a search elsewhere for the causes of untoward clinical reactions which have been reported (14).

On the cat, 1.19 ± 0.06 Gm. of *ISD* (1936) is equivalent to 1 Gm. of *USP RDP* or slightly less than the equivalence by any of the frog methods. Calculated as above, it is seen that in the cat, 0.067 Gm. of *USP RDP* exhibits the activity of 1 International Digitalis Unit and when determined on this species the discrepancy between the *USP* and international units is only 10%. Making allowance for the relationship actually observed between the two international standards, which indicates that 0.0862 Gm. of the *ISD* (1936) represents 1 International Digitalis Unit in the cat (13), the discrepancy between the latter unit and the *USP* unit is only 3% and entirely negligible.

Potency of the Study Powder.[5]—The study powder has been compared against the *USP* Standard Preparation of Digitalis (containing 0.0745 Gm. of *USP RDP* per cc.) in assays by each of three methods. Eighty frogs were used in each of the *USP* XI assays while 77, 90, 80 and 90 frogs were used, respectively, in the 18-hr. assays listed in that order in Table II. The three cat assays represent the use of 12, 17 and 20 cats, respectively. The results, listed in Table II, have all been expressed in terms of the volume of the standard preparation which was equivalent to 0.1 Gm. of the original study powder. It will be noted that the agreement between the results of individual assays by the same method is quite satisfactory. Although there is a slight indication of higher potency in the assay conducted in 1940 by the *USP* XI method, this value is not significantly higher than those obtained later. The agreement in general between the various assays by the same method at different times assumes added significance when it is considered that the data represent determinations made independently by each writer using each of the three methods. With one exception, the *USP* XI assays of November 26, 1941, and December 1, 1941, no two assays were made on the same pair of standard tinctures.

The data in Table II reveal that the results of the 1-hr. and 18-hr. methods on frogs agree very well,

[5] As used throughout this paper the term "study powder" refers exclusively to the material distributed as samples 3 and 5, respectively, for the second and third comparisons (using frogs) of the *USP* (1939–1941) digitalis study. Actually only the 1940 assays reported in Table II were conducted on the study powder in its original concentration. Developments discussed elsewhere (15) resulted in a stepwise dilution of the study powder in the proportion of 1 : 1.351 to yield what is now the *USP* Digitalis Reference Standard (1942). Information on this dilution has been obtained through the courtesy of Professor E. Fullerton Cook making it possible to adjust the results of the 1941 and 1942 assays to the basis of the original study powder.

TABLE II.—SUMMARY OF ASSAYS OF THE STUDY POWDER AGAINST *USP* STANDARD PREPARATION OF DIGITALIS

USP XI Method			18-Hr. Frog Method			*USP* XII Method		
	Potency[a]			Potency[a]			Potency[a]	
Date	Cc.	Log ± *S. E.*	Date	Cc.	Log ± *S. E.*	Date	Cc.	Log ± *S. E.*
May 8, 1940	1.20	0.0779 ± 0.0539	July 2, 1940	1.09	0.0374 ± 0.0226	Sept. 29, 1941	1.13	0.0544 ± 0.0283
Nov. 26, 1941	1.06	0.0241 ± 0.0548	July 15, 1940	0.90	−.0461 ± 0.0276	Oct. 31, 1941	1.16	0.0637 ± 0.0256
Dec. 1, 1941	1.01	0.0053 ± 0.0563	July 30, 1940	1.02	0.0075 ± 0.0409	May 29, 1942	1.13	0.0528 ± 0.0507
Dec. 4, 1941	1.00	−.0019 ± 0.0556	June 8, 1942	1.01	0.0044 ± 0.0410			
Weighted mean	1.06	0.0273 ± 0.0276		1.01	0.0044 ± 0.0150		1.14	0.0585 ±0.0178

[a] The potency value given in columns 2, 5 and 8 is the volume of *USP* XI Standard Preparation of Digitalis (containing the activity of 0.0745 Gm. of *USP* Reference Digitalis powder per cc.) equivalent to 0.1 Gm. of the original study powder; this value, multiplied by 100, gives the potency of the study powder expressed as a percentage of the *USP* XI requirements. The values given in columns 3, 6 and 9 are the logarithms of the volume and its standard error, respectively, which are conventionally known as $M \pm s_M$ (10).

TABLE III.—*USP* XI AND *USP* XII POTENCIES OF SEVERAL SAMPLES OF DIGITALIS

Sample No.	Type of Preparation	Potency[a] *USP* XI Method, Cc.	Potency[a] *USP* XII Method, Cc.	Ratio of *USP* XII to XI	*USP* XII Potency[b]	Excess of *USP* XI over *USP* XII *USP* Digitalis Units[c]
7707	Tincture	1.49 ± 0.08	1.77 ± 0.20	1.19 ± 0.16	2.11	0.42 ± 0.19
7935		1.72 ± 0.08	2.15 ± 0.14	1.25 ± 0.10	2.56	0.49 ± 0.12
8088		0.57 ± 0.04	1.01 ± 0.06	1.77 ± 0.16	1.20	1.11 ± 0.19
8228		0.52 ± 0.06	0.75 ± 0.03	1.42 ± 0.17	0.89	0.69 ± 0.21
8429		1.58 ± 0.12	2.32 ± 0.10	1.46 ± 0.12	2.76	0.74 ± 0.15
8435		1.30 ± 0.14	1.42 ± 0.08	1.09 ± 0.14	1.69	0.30 ± 0.16
8474		0.88 ± 0.08	1.02 ± 0.05	1.16 ± 0.12	1.21	0.38 ± 0.14
8502		1.58 ± 0.10	2.35 ± 0.16	1.48 ± 0.13	2.80	0.77 ± 0.16
Study	Powder	1.06 ± 0.07	1.14 ± 0.05	1.07 ± 0.08	1.35	0.28 ± 0.10
ISD (1936)		1.06 ± 0.03	1.13 ± 0.04	1.07 ± 0.06	1.35	0.27 ± 0.07
7556		0.57 ± 0.05	0.82 ± 0.06	1.45 ± 0.16	0.92	0.73 ± 0.19
7608		1.07 ± 0.11	0.97 ± 0.06	0.91 ± 0.11	1.20	0.09 ± 0.16
8229		0.53 ± 0.07	0.70 ± 0.07	1.33 ± 0.21	0.83	0.59 ± 0.26
8000	Leaves	1.28 ± 0.11	1.10 ± 0.07	0.86 ± 0.09	1.31	0.02 ± 0.15
7171	Tablet	0.44 ± 0.03	0.62 ± 0.04	1.40 ± 0.14	0.74	0.66 ± 0.17
7967		0.32 ± 0.02	0.51 ± 0.04	1.59 ± 0.16	0.61	0.89 ± 0.19
7873	Powdered	1.15 ± 0.09	1.66 ± 0.07	1.44 ± 0.13	1.98	0.72 ± 0.15
7893	extract	1.55 ± 0.12	2.32 ± 0.18	1.49 ± 0.16	2.76	0.78 ± 0.19

[a] The potency values in columns 3 and 4 are expressed in terms of the volume of *USP* Standard Preparation (containing 0.0745 Gm. of *USP RDP* per cc.) to which 1 cc. of tincture (or 0.1 Gm. of powder, whole leaf or powdered extract) is equivalent by the method of assay indicated.

[b] Calculated by multiplying data in column 4 by 1.19.

[c] The potency values in columns 6 and 7 are expressed in *USP* Digitalis Units, each of which represents 0.1 Gm. of *USP* Digitalis Reference Standard (1942).

the difference between them being clearly insignificant. A weighted average of all the data obtained on frogs indicates that 0.1 Gm. of the study powder was equivalent to 1.02 ± 0.03 cc. of the *USP* Standard Preparation of Digitalis, each cc. of which represented 0.0745 Gm. of *USP RDP*. It is thus seen that, as originally blended, the study powder met exactly the potency requirements of *USP* XI.

On cats, the study powder exhibited a slightly greater relative effect than the *USP* Standard Preparation, the weighted average being 114% as compared with 102% on frogs. From this determination on cats it is possible to calculate the relation between the *USP RDP*, which was available from the time of its release early in 1936 until recently, and the *USP DRS* which is the new digitalis standard for the United States. The latter should exhibit 74% (1/1.351) of its original potency.[6] Thus, it should establish the potency of *USP* XII digitalis at about 84% (0.74 × 114) of the standard of strength of *USP* XI, a reduction of about 16%. This reduction may be looked upon as the decrement attributable to the change in reference standards and is, of course, a fixed amount. That it is not the total reduction is indicated in Table III, from which it is clear that an additional decrement results from changing the test animal.

Relative Potency of USP XI and USP XII Digitalis.—In view of statements in the literature (16, 17) tending to discredit the use of frogs in assaying digitalis and, finally, the action of the *USP* Committee of Revision in adopting a method based on cats for *USP* XII, it was of great interest to compare the results of assays of the same samples by the *USP* XI and *USP* XII methods. Since the useful-

[6] It is a curious coincidence that had the study powder been adopted in its original potency as a new reference standard to be used with the *USP* XII method, the necessary factor would have been almost exactly 0.745, that currently in force for *USP RDP*. The desirability of dispensing with the somewhat confusing factor is manifest.

ness of such a comparison depends solely upon the reliability of the respective determinations involved, the basic data in Table III were extended to the limits of practicality. In column 2 is indicated the type of the preparations identified by numbers in column 1. Column 3 indicates the *USP* XI potency of each preparation and the average standard error of the estimate. As pointed out in the introduction, the *USP* XI potencies of many of these preparations were such as to indicate regulatory action. The series of tinctures includes both subpotent and overstrength samples, the potency values of which range from 52% to 172% *USP* XI. The variation in potency of the six samples of powdered digitalis or whole leaf is less extreme; three of the powders meet the *USP* XI requirements exactly although only one of the three (7608) is a standardized product. Sample 7556 is the much-studied (16, 17, 18) New York Heart Association Powder No. 7.[7] Sample 8229, listed as a powder, was submitted in capsule form. The two samples of tablet preparations reported show a very low potency by the official method. Assays were made of these on cats since each of the manufacturers involved claimed that his product exhibited full potency upon cats. The degree to which these claims are substantiated is seen in the data. It is of interest to note that sample 7967 was purported to consist of *Digitalis lanata*.

Column 4 lists the potency indicated by each preparation when compared by the *USP* XII technique against the same standard used in the *USP* XI method. A comparison of the data in columns 3 and 4 reveals, as indicated by the ratios listed in column 5, that the activity exhibited by the *USP* XII method (on cats) is generally greater than that shown by the *USP* XI method (on frogs). This species difference in response to the same preparation is such that nine of the eighteen samples examined appear at least 40% stronger on the cat than on the frog. Thus, if the new reference standard were fully as active (on cats) as the reference standard heretofore available and if these preparations met the *USP* XI requirements exactly, their greater relative activity on the cat would make it necessary to dilute them at least 30% to bring them into compliance with the *USP* XII requirements. As a matter of fact, few of them are of *USP* XI strength so that in comparison with the weaker reference standard the overstrength preparations greatly exceed the *USP* XII potency, while those markedly deficient by *USP* XI standards come close to meeting the *USP* XII standards. This is clearly indicated in column 6 which gives the potency of the samples in terms of the *USP* XII standard of strength as calculated from the values in column 4 coupled with the information presented in Table II that 1 cc. of *USP* Standard Preparation (of *USP RDP*) represents the activity of 1.19 (1/0.84) cc. of *USP* XII Tincture of Digitalis.

[7] A quantity of New York Heart Association Digitalis Powder No. 7 was obtained through the kindness of Dr. Harry Gold, Cornell Medical College.

The ratios listed in column 5 vary considerably with no apparent relation to either the absolute level of potency or the type of preparation. However, the highest ratios are observed in the tinctures and generally lower ratios prevail among the powders. Of the latter, samples 7556 and 8229 are grayish brown in color, the chlorophyll having faded noticeably. The ratios of these samples are slightly higher than those of the study powder and *ISD* (1936) which powders, of course, have been carefully preserved so that the chlorophyll is still bright green. Samples 7608 and 8000, which are English digitalis, are also bright green and are unique in showing less activity on the cat than on the frog. The observations on powders are obviously too scanty to support any speculation, but it may prove significant that the highest cat-to-frog potency ratios are seen in the two powders which are definitely "off-color" and subpotent by *USP* XI standards. The two samples of powdered extract examined were also definitely subpotent since 0.1 Gm. should be equivalent to at least 2.75 cc. of *USP* Standard Preparation of Digitalis (19).[8] It is obvious, of course, that these findings are in harmony with the view generally held (20) with respect to deterioration in these products, *i. e.*, that their activity on the frog decreases while that on the cat remains practically unchanged.

All of the tinctures in the series are known to be several months old. They have been stored under refrigeration since their receipt in the laboratory and some of them have been reassayed by the *USP* XI method on several occasions. Sample 7707, for example, was assayed at 12 and 18 months after the initial series of assays. In no case has any evidence of a loss in potency been observed under these conditions. No correlation between the known age of the tinctures and the cat-to-frog ratio of potencies is evident in this limited series. It is regrettable that circumstances preclude detailed studies of the histories of the samples which might lead to helpful generalizations. Unfortunately, also, the variation in the cat-to-frog ratio is so great from one tincture to another that it precludes the possibility of striking an average that might satisfactorily represent the ratio of *USP* XII to *USP* XI standards of potency for even this dosage form of digitalis. Statistical analysis indicates that there is less than one chance in a hundred that the difference in the ratios for these specimens of tincture is due simply to sampling error. This is forcibly shown in the data in column 7 of Table III. These values indicate the extent to which these various specimens of digitalis would exceed the *USP* XII requirements if all were adjusted to 100% *USP* XI. The excess varies from over 1 *USP* Digitalis Unit (as represented by the new reference standard) to practically zero in those cases where the calculated excess is less than twice the standard error.

[8] In order to simplify the discussion of the powdered extracts, which are official National Formulary items, their potencies are expressed throughout in terms of *USP* requirements.

With respect to appraising the change in potency of digitalis preparations resulting from the revision of the standards, the leeway or allowed tolerance in standardizing to *USP* XI requirements must be taken into consideration. Thus, if a *USP* XI Tincture of Digitalis having a potency of 0.8 *USP* Digitalis Unit per cc. were considered acceptable, it is seen that its potency might very well be practically that required for *USP* XII Tincture of Digitalis. If, however, the *USP* XI product exhibited in the frog a potency of 1.32 *USP* Digitalis Units per cc., it would be expected to show, per cc., at least 1.5 and possibly 2.6 Digitalis Units as these are defined under *USP* XII. In order to adjust such a preparation to the *USP* XII standard of strength, a very substantial reduction is necessary.

It will be noted that the range of values in both columns 3 and 5 is such that the strongest is three times as potent as the weakest. A similar spread between the weakest and strongest of a series of commercial digitalis tinctures has been reported by Gold and his co-workers (16, 17, 21) as evidence of variation in the potency manifested in the cat in samples presumed to meet the *USP* XI requirements. While this presumption may be correct, it is hardly likely, in view of the data of Table III, that all of them were of the *same* potency by any method of assay. It is not widely realized that two samples differing as much as 65% in strength might both be considered as meeting the *USP* XI requirements under a liberal interpretation of the footnote on page 398, *USP* XI, which reads "Owing to many variable factors in the standardization of digitalis, evidence of potency in all digitalis assays to within 20 per cent above or 20 per cent below the standard, is acceptable."

In this connection, it is worthy of note that the *USP* XII assay provides for a ±20% tolerance and at the same time defines what is acceptable in the way of evidence that the tolerance has not been exceeded. The potency of the preparation being assayed is expressed in the customary manner as the ratio of the respective average lethal doses of standard and unknown. To insure satisfactory accuracy in this ratio, an upper limit has been set upon the allowable variation in the determination of each average lethal dose, the extent of the variation being calculated as the standard error. If this limit on the individual standard errors of ±5.7% is not exceeded, the standard error of the ratio will not exceed ±10% of the ratio. By virtue of the conventional definition of the standard error (22), it follows then that the chances are only one in twenty that the observed ratio differs from the true ratio by as much as ±20%. As applied to Tincture of Digitalis, *USP* XII, this provision affords a basis for confidence that the actual potency will not differ by more than ±0.2 *USP* Digitalis Unit per cc. from the prescribed potency of 1.0 unit per cc. It should be noted that if the accuracy is just sufficient to meet the *USP* XI requirements, *i. e.*, that the calculated standard errors are each just 5.7%, subsequent adjustment of the potency to either more or less than 1.0 unit per cc. of tincture involves the risk of having the finished product vary more than ±0.2 unit from the requirement (23) that "1 cc. shall be equivalent to 1.0 *USP* Digitalis Unit." A tincture adjusted to a potency of 0.85 unit per cc. on the basis of such an assay would scarcely be an acceptable *USP* XII product since it can be shown that the likelihood that its true potency is 1.0 unit per cc. is very much less than that of its being less than 0.8 unit per cc. or 20% below the requirement. Under *USP* XI, however, such a tincture presumably would have been satisfactory since there is "evidence of potency . . . . to within . . . . 20 per cent below the standard." It is clear, of course, that by achieving greater accuracy in the assay (by using more cats or by adopting means of reducing the variability in their responses) the amount of likely variation may be reduced to materially less than ±0.2 unit. The new requirements will demand more careful standardization of this class of drugs in which the need for accurate dosage is second only to that for insulin.

Comparison of the Potencies of Digitalis Preparations Indicated by the USP XI and 18-Hr. Methods.— For more than three decades the question of the length of time to be allowed for injected digitalis to react in assays on frogs has been a live issue (3, 5, 24). Until recently, attention was focused upon the completeness of absorption attained in the assay rather than upon the influence of variations in the length of the assay upon the relative potency of the preparations being assayed. In view of the lack of conclusive evidence on the latter point, two comparisons of the *USP* (1939–1941) digitalis study (15) were devoted to concurrent assays on frogs of the same material by the two methods most widely used which depend, respectively, upon observations for 1 hour and 18 hours. The conclusion was reached (12) that the two methods gave substantially the same result. In arriving at this conclusion little consideration was paid to the fact that the materials studied were good quality powdered digitalis. Importance of the type of preparation in this respect is brought out in Table IV. This table is similar to Table III in arrangement and content except that a comparison is drawn between the ratios of assays by both the *USP* XI and 18-hr. methods. The data designated as the "first series" were obtained in 1938–1939 before the details of the 18-hr. method had been standardized to the extent accomplished later in connection with the *USP* (1939–1941) digitalis study (15). With few exceptions the assays in this series were carried out by both methods on the same day using frogs from the same batch. Hence, there was no question of a change in either the sample or in the frogs between assays by the two methods. Column 5 of Table IV lists the ratios of the potencies observed by the 18-hr. and the *USP* XI method, respectively. It will be noted that in general this ratio is higher for the samples of leaf than for the samples of tincture; the one sample of fluidextract examined behaved like the tinctures. This pointed

TABLE IV.—COMPARISON OF THE POTENCIES OF DIGITALIS PREPARATIONS INDICATED BY THE *USP* XI AND THE 18-HR. METHODS

Sample No.	Type of Preparation	Potency[a] *USP* XI Method, Cc.	Potency[a] 18-Hr. Method, Cc.	18-Hr./*USP* XI Ratio ± *S. E.*
		FIRST SERIES		
6817	Tincture	0.78 ± 0.14[b]	0.58 ± 0.06	0.74 ± 0.15
6837		0.58 ± 0.04	0.47 ± 0.02[c]	0.81 ± 0.07
6869		0.66 ± 0.08	0.63 ± 0.05	0.95 ± 0.14
6881		1.23 ± 0.24	1.00 ± 0.07[b]	0.81 ± 0.17
6883		1.13 ± 0.18	0.68 ± 0.05	0.60 ± 0.11
6816	Fluidextract	0.49 ± 0.05[c]	0.38 ± 0.02	0.78 ± 0.10
6885	Leaves	0.88 ± 0.18	0.83 ± 0.08	0.94 ± 0.21
6886		1.30 ± 0.30	1.04 ± 0.06	0.80 ± 0.19
6898		1.00 ± 0.20	1.05 ± 0.05	1.05 ± 0.22
6927		0.96 ± 0.15	0.94 ± 0.06	0.98 ± 0.17
6857	Capsules	0.91 ± 0.13	0.73 ± 0.03[c]	0.80 ± 0.12
6855	Tablets	0.61 ± 0.10	0.42 ± 0.06	0.69 ± 0.15
		SECOND SERIES		
7707	Tincture	1.49 ± 0.08	0.99 ± 0.06	0.67 ± 0.05
7935		1.72 ± 0.08	1.30 ± 0.07	0.76 ± 0.06
8088		0.57 ± 0.04	0.45 ± 0.03	0.80 ± 0.07
8228		0.52 ± 0.06	0.48 ± 0.02	0.90 ± 0.11
8429		1.58 ± 0.12	1.50 ± 0.07	0.95 ± 0.08
8435		1.30 ± 0.14	0.90 ± 0.07	0.69 ± 0.09
8474		0.88 ± 0.08	0.54 ± 0.03	0.62 ± 0.06
8502		1.58 ± 0.10	1.17 ± 0.08	0.74 ± 0.07
Study	Powder	1.06 ± 0.07	1.01 ± 0.04	0.95 ± 0.07
ISD (1936)		1.06 ± 0.03	1.06 ± 0.02	1.00 ± 0.03
7556		0.57 ± 0.05	0.60 ± 0.03	1.07 ± 0.10
7608		1.07 ± 0.11	1.03 ± 0.05	0.97 ± 0.11
8229		0.53 ± 0.07	0.53 ± 0.03	1.00 ± 0.14
8000	Leaves	1.28 ± 0.11	1.28 ± 0.06	1.00 ± 0.10
7171	Tablet	0.44 ± 0.03	0.55 ± 0.05	1.24 ± 0.14
7967		0.32 ± 0.02	0.31 ± 0.03	0.96 ± 0.10
7873	Powdered	1.15 ± 0.09	0.88 ± 0.08	0.77 ± 0.09
7893	extract	1.55 ± 0.12	1.34 ± 0.06	0.87 ± 0.08

[a] The potency values in columns 3 and 4 are expressed in terms of the volume of *USP* Standard Preparation (containing 0.0745 Gm. of *USP RDP* per cc.) to which 1 cc. of tincture (or 0.1 Gm. of powder, whole leaf or powdered extract) is equivalent by the method of assay indicated. Unless otherwise indicated, the values in the first series represent single assays. In the second series, each value represents two or more assays.

[b] Weighted average of three assays.

[c] Weighted average of two assays.

to a definite trend in favor of the conclusion that the two methods give the same potency for samples of leaves, but not for liquid preparations. Such a conclusion is borne out by a critical statistical examination which indicates that the differences observed in this series would occur by chance only 3 times in 200 if no difference actually existed between leaves and tincture in this respect. A similar discrepancy between the potency of a tincture indicated by the *USP* XI and overnight methods was noted in the A. PH. A. cooperative assays (4).

The second series of assays completed recently on the same samples listed in Table III adds sufficient further evidence to demonstrate conclusively the difference between the results of assays by the two methods on the two popular dosage forms of digitalis. In this series the assays were not carried out strictly simultaneously, but sufficient checks were made to insure that the differences observed could not be due to a change in potency between the time of the USP XI and 18-hr. assays. Further, in this series no less than two assays of each sample were made by each method so that the individual data are much more reliable for establishing the existence of a discrepancy between the two methods than are the data of the first series.

The powders studied in the second series are not representative of the powdered digitalis generally available since two of the six specimens were reference standards. The data, therefore, are obviously too scanty to support generalization, but it is interesting to note that the two powders which show the highest cat-to-frog potency ratios have identical potencies by the *USP* XI and 18-hr. methods. The two samples of powdered extract give ratios characteristic of tinctures, an observation which probably reflects their kinship to tinctures in mode of preparation.

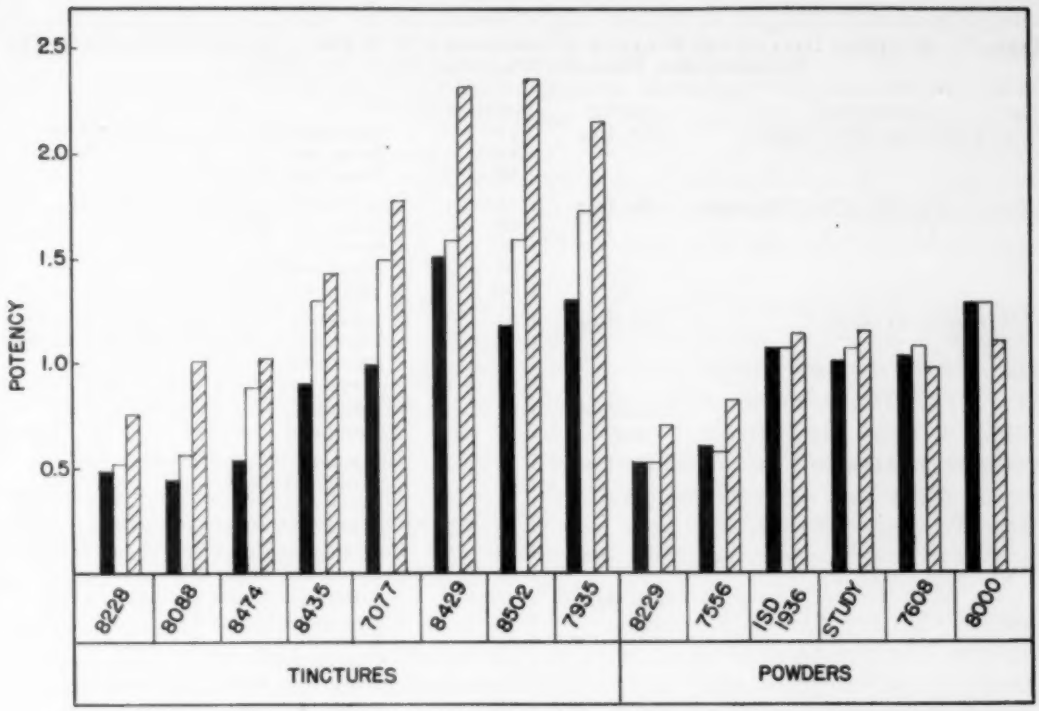

Figure 1.

The vertical bars represent the potency, determined as indicated and expressed on the ordinate as cc. of *USP* Standard Preparation of Digitalis (each cc. representing 0.0745 Gm. of *USP* Reference Digitalis Powder), of the samples identified by number along the bottom of the graph. Solid bars, 18-hr. method; open bars, *USP* XI method; hatched bars, *USP* XII method. Data taken from Tables III and IV.

The USP Digitalis Unit.—Figure 1 brings together the essential data of Tables III and IV in order to provide a graphic comparison of the results of the three methods of assay as applied to tinctures and powders. If it is true that assays on cats of the galenical preparations are more indicative of the true therapeutic efficacy (17), it is obvious that adoption of the 18-hr. method would have materially increased the discrepancy between biological assay and human response in the case of many preparations. Furthermore, it is clear that there is sufficient difference between the results of official assays and those on cats to afford ample basis, quite apart from variations in the *USP* XI potency, for the reports (14) of digitalis overdosage. The physician's difficulties cannot be attributed wholly to the discrepancy between the species of animals, however, since a substantial proportion of the variation in potency from one brand of digitalis to another might be traced to the original standardization.

It may be true that refinements in assay technique and slight variations in potency are of more concern to the biological assayist than to the physician, but it is important to the latter to be able universally to procure digitalis of uniform potency. A complaint was voiced recently (25) against the practice of expressing digitalis potency in units on the grounds that "the dose for each (patient) is best considered in grains or in minims, regardless of the method used for standardization." This statement neglects entirely two facts: (*a*) The native activity of digitalis varies widely from specimen to specimen, and (*b*), as illustrated in Fig. 1, the method of standardization is of paramount importance in determining the activity. It is unquestionably desirable to eliminate the possibility of confusion by limiting the variety of expressions for denoting digitalis activity. However, this depends primarily upon general acceptance by the medical profession of some expression of activity such as the *USP* Digitalis Unit.

As has been pointed out to the medical profession by Nelson (26) the absolute potency of the unit of digitalis activity that will prevail under the *USP* XII standards of strength is less than that fixed by the *USP* XI standards. The reduction is the result of changing not only the reference standard but also the test animal. *USP* XII Digitalis will be adjusted, on the basis of the new assay procedure, to the potency established by the International Standard Digitalis (1936). As emphasized by Nelson, and as may be calculated from Table V, the USP XII preparations will be about 25% stronger than those of *USP* X. This holds true for products which exhibit the same relative activity in both cats and frogs. The data of this paper show that for such preparations the *USP* XII potency will be about 16% weaker than that of *USP* XI. In the case of those preparations which exhibit a substantially greater activity on the cat than on the frog the data

TABLE V.—COMPILED DATA ON THE RELATIVE POTENCIES OF *USP* X DIGITALIS, *USP* XI DIGITALIS AND INTERNATIONAL DIGITALIS STANDARDS (1926) AND (1936)

Comparison	Method	Potency Ratio	Observer
USP X Digitalis/*ISD* (1926)	1-Hr. frog	0.77	Edmunds, Lovell and Braden (30)
		0.81	Rowe (31)
		0.83	Rowe and Pfeifle (5)
USP XI Digitalis/*USP* X Digitalis	1-Hr. frog	1.55	A. PH. A. cooperative study (4)
		1.52	Rowe and Pfeifle (5)
		1.40	Rowe (6)
		1.53	Ichniowski and Thompson (7)
		1.60	Swoap and Pabst (8)
USP RDP/ISD (1926)	1-Hr. frog	1.34	Edmunds, Moyer and Shaw (3)
		1.35	Chen (reported by Edmunds, *et al.* (3))
		1.81	Rowe and Pfeifle (5)
		2.07 ± 0.08	Braun and Miller (this paper)
	Overnight frog	1.91	Edmunds, Moyer and Shaw (3)
		1.91	Rowe and Pfeifle (5)
USP RDP/ISD (1936)	1-Hr. frog	1.13	Edmunds, Moyer and Shaw (3)
		1.18	Rowe and Pfeifle (5)
		1.41	Rowe (6)
		1.27 ± 0.04	Braun and Miller (this paper)
	4-Hr. frog	1.19	Edmunds, Moyer and Shaw (3)
		1.38 ± 0.10	Braun and Miller (this paper)
	Overnight frog	1.36	Edmunds, Moyer and Shaw (3)
		1.18	Rowe and Pfeifle (5)
		1.48	Rowe (6)
		1.22	Chapman (32)
		1.27 ± 0.02	Braun and Miller (this paper)
	Cat	1.16	Edmunds, Moyer and Shaw (3)
		1.19 ± 0.06	Braun and Miller (this paper)

show that in meeting the *USP* XII requirements the potency may not be much more than half that required under *USP* XI. This is not to be interpreted as evidence of fluctation in the *USP* Digitalis Unit, which was fixed under *USP* XI as the activity on frogs of 0.0745 Gm. of *USP RDP* and under *USP* XII (as a result of a coincidence) as the activity on cats of 0.1 Gm. of *USP DRS*. The variability is resident in the somewhat unpredictable characteristics of the digitalis preparations being standardized.

The question will arise in the minds of many as to why the time-honored expression "cat unit" should be abandoned in favor of the *USP* Digitalis Unit. The reasons have been set forth quite adequately by Burn (27) who has marshaled the arguments against the use of units defined in terms of physiological effects. While there will be limitations on even the *USP* unit in determining the human dosage of the purified glycosides (28), the way is open for avoiding potency differences between laboratories due to variations in determinations of the fatal dose of digitalis to cats as well as similar differences due to variations within the confines of a single laboratory. As demonstrated recently in carefully controlled experiments (29) the latter can assume quite significant proportions. Until a chemical method of assay is developed for digitalis, it will be the responsibility of the biological assayist to insure uniformity in potency from one lot to another of each digitalis preparation. The correct dosage in *USP* Digitalis Units of each type of preparation can be determined only by clinical investigation and experience.

Compiled Data on the Relative Potencies of USP X Digitalis, USP XI Digitalis and International Digitalis Standards (1926) and (1936).—Table V lists a number of values compiled from the literature indicating the relationship between the *USP* X and *USP* XI standards of strength for digitalis and certain comparisons of these with the two international standards (1926) and (1936). The investigative effort represented by the data compiled in Table V is truly monumental and yet by no means does it indicate the full amount of research conducted on the assay of digitalis in the past decade. Unfortunately, there is a great deal of variation in the amount of experimental work supporting the various values and no attempt has been made to determine their inherent reliability. This might be done by calculating the respective standard errors as has been done for the data in this paper. In the light of present information on the assay of digitalis, however, it is known that many of the ratios in Table V represent so few experimental observations that they can be little more than rough approximations of the true relationships. In spite of this, there is a striking agreement throughout the series with one notable exception, namely, the comparison of the *USP RDP* against *ISD* (1926). It is a regrettable circumstance that this comparison of such critical importance should have proved to be the only controversial one. Had almost any of the other comparisons listed in Table V been made the basis of establishing the potency of *USP* XI Digitalis, a great deal of subsequent research and journal space could have been directed into more profitable channels. No explanation of the discrepancy in

these determinations is apparent. Of the possible explanations which have been explored in this laboratory, such as the influence of alcoholic content of the test dilutions and possible heterogeneity of the ampuls of *ISD* (1926), none has given any basis for the variation observed. In view of recent developments the question becomes one solely of academic importance with little promise of a satisfactory solution.

SUMMARY

1. By the *USP* XI assay, the *USP* Reference Digitalis Powder which was released in 1936 was found to be 2.07 ± 0.08 times as potent as International Standard Digitalis (1926).

2. By the *USP* XI assay and assays on frogs involving longer periods of observation than one hour, the *USP* Reference Digitalis Powder was found to be 1.27 ± 0.02 times as potent as International Standard Digitalis (1936) and in assays on cats by the *USP* XII procedure, it was found to be 1.19 ± 0.06 times as potent as International Standard Digitalis (1936).

3. In assays on frogs by the *USP* XI and 18-hr. methods, the *USP* study powder was found to meet almost exactly the potency requirements for *USP* XI Powdered Digitalis, while in assays on cats the potency observed was 114% of that required for powdered digitalis of *USP* XI strength. Since the *USP* Digitalis Reference Standard (1942) is a dilution of the *USP* study powder, these data indicate that insofar as can be determined by the *USP* XII method of assay, the *USP* XII standard of potency will equal that established by International Standard Digitalis (1936) and will be about 16% weaker than the *USP* XI standard of potency.

4. The *USP* XI and *USP* XII potencies of several samples of the various pharmaceutical forms of digitalis were determined. The data show that complying with the revised standards of strength will result in a substantial reduction in the potency to which the American physician has become accustomed during the past six years. The indication is that the change may be greater in tinctures than in powdered digitalis.

5. Assays on frogs by the *USP* XI and 18-hr. methods gave substantially identical results with digitalis leaves and powdered digitalis; with tinctures, the potency indicated by the 18-hr. method is significantly lower than that shown by the *USP* XI assay.

6. A review is presented of the published data on the relative potencies of *USP* X Digitalis, *USP* XI Digitalis and International Digitalis Standards (1926) and (1936).

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Comparison of Digoxin, Digilanids *A*, *B* and *C*, and Deacetyldigilanids *A* and *B**†

By K. K. Chen, Chester C. Hargreaves and E. Brown Robbins

After *Digitalis lanata* was shown to have more physiologic units quantitatively than *Digitalis purpurea* (1), chemical investigations were carried out in a few laboratories. The most pertinent question to settle is whether or not the active constituents of one species are identical with those of the other. Smith (2, 3) succeeded in isolating digoxin and gitoxin, and studied the constitution of the aglycone of digoxin, digoxigenin (4). Mannich and his associates (5) announced the separation of lanata-glycosides I, II, III and IV, and later made several revisions (6, 7, 8) in the light of other workers' findings. Much systematic information has been furnished by Stoll, Kreis and Hofmann (9, 10, 11, 12, 13, 14, 15, 16). According to their results, the leaves of *Digitalis lanata* contain three polysides, digilanids *A*, *B* and *C*. The latter under suitable conditions of hydrolysis give rise to glycosides of smaller molecular sizes as follows:

Digilanid *A* → Deacetyldigilanid *A* + CH_3COOH
↓
Digitoxin + $C_6H_{12}O_6$ (glucose)
↓
Digitoxigenin + 3 $C_6H_{12}O_4$ (digitoxose)

Digilanid *B* → Deacetyldigilanid *B* + CH_3COOH
↓
Gitoxin + $C_6H_{12}O_6$
↓
Gitoxigenin + 3 $C_6H_{12}O_4$

Digilanid *C* → Deacetyldigilanid *C* + CH_3COOH
↓
Digoxin + $C_6H_{12}O_6$
↓
Digoxigenin + 3 $C_6H_{12}O_4$

Interestingly, Stoll and Kreis (17) have shown that deacetyldigilanids *A* and *B* also occur in the leaves of *Digitalis purpurea*, although, heretofore, digitoxin and gitoxin have been long considered as the main crystalline principles of this well-known plant (18). The simpler glycosides of digilanid *C*, namely, deacetyldigilanid *C* and digoxin, are apparently absent in the leaves of *D. purpurea*.

The potencies of the above compounds have been repeatedly determined. The results as published by different workers are compiled in Table I. The discrepancies are undoubtedly due to many factors such as the strain of animals, the atmospheric temperature and variations in experimental procedures. The anesthetic in cats exerts a great influence on the outcome of tests, as is well illustrated by Kaplan and Visscher (23). Digoxin has been assayed on guinea pigs, rabbits, pigeons and dogs (23, 26, 28, 29). Quantitative evaluation of digoxin and digilanid *C* can also be made on the embryonic chick heart (30). Moe and Visscher (22), by means of the heart-lung preparation, presented evidence that digilanid *C* was especially effective in increasing the cardiac efficiency in relatively small doses, as compared with digilanids *A* and *B*. This has been questioned by Cattell and Gold (31).

The present study deals with the evaluation of digoxin, digilanids *A*, *B* and *C*, and deacetyldigilanids *A* and *B*, in cats and frogs under as nearly comparable conditions as possible. The results so obtained furnish secure grounds for correct assignment of the order of activity. Besides, they reliably demonstrate the significance of the

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TABLE I.—PUBLISHED DATA ON FROGS AND CATS

Compound	Cat Lethal Dose		Frog Lethal Dose	
	Mg./Kg.	Author	Mg./Gm.	Author
Digilanid *A*	0.380	Rothlin (19, 20, 21)	0.00145	Rothlin (20)
	0.45	Moe and Visscher (22)		
	0.32	Kaplan and Visscher (23)		
	0.368	Rothlin (12)		
Digilanid *B*	0.403	Rothlin (19, 20, 21)	0.00185	Rothlin (20)
	0.65	Moe and Visscher (22)		
	0.58	Kaplan and Visscher (23)		
	0.40	Kaplan and Visscher (23)		
	0.346	Rothlin (12)		
Digilanid *C*	0.280	Rothlin (19, 20, 21)	0.00156	Rothlin (20)
	0.390	Moe and Visscher (22)	0.0016	Kwit, Gold and Cattell (24)
	0.34	Kaplan and Visscher (23)		
	0.23	Kaplan and Visscher (23)		
	0.255	Rothlin (12)		
	0.29	Kwit, Gold and Cattell (24)		
	0.261	DeGraff and Lehman (25)		
Deacetyldigilanid *A*	0.337	Rothlin (12)	0.00145	Rothlin (15, 16)
	0.368	Rothlin (15, 16)		
Deacetyldigilanid *B*	0.228	Rothlin (12)	0.00318	Rothlin (15, 16)
	0.369	Rothlin (15, 16)		
Deacetyldigilanid *C*	0.228	Rothlin (12)		
Digoxin	0.442	White (26)	1 mg. = 0.28 mg. of ouabain	Trevan (2)
	0.22[a]	Chen, Chen and Anderson (27)	0.0025	Chen, Chen and Anderson (27)
	0.424	Walker (28)	0.00154	Rothlin (15, 16)
	0.335	DeGraff and Lehman (25)		
	0.280	Rothlin (21)		

[a] ±0.008.

acetyl and glucose radicals upon the potency. With the exception of digoxin, which was courteously supplied by Burroughs Wellcome and Company, Tuckahoe, New York, all the glycosides were provided by Dr. Arthur Stoll of Basel, Switzerland.

EXPERIMENTAL

To effect 0.1% stock solutions, each compound was dissolved in 47.5% ethanol (by volume), except deacetyldigilanid *B* which required 57% ethanol. Frog assays were made according to the U. S. P. (32) with dilutions of 1:4000 and 1:2000; and cat assays, according to procedures previously published (27,

TABLE II.—ASSAY IN CATS

Compound	Sex of Cat	Body Weight, Kg.	Heart Weight, Gm.	Fatal Dose by Body Weight, μg./Kg.	Fatal Dose by Heart Weight, μg./Gm.	Mean Lethal Dose by Body Weight, μg./Kg.	Mean Lethal Dose by Heart Weight, μg./Gm.
Digilanid *A*	*M*	2.702	8.4	288.7	92.9	361.0 ± 17.25	97.2 ± 4.99
	F	2.037	8.9	437.9	100.2		
	M	2.073	7.3	405.2	115.1		
	F	1.741	5.9	399.5	117.9		
	F	2.733	10.5	337.0	87.7		
	M	2.594	11.1	327.7	76.6		
	F	2.531	10.4	370.6	90.2		
	F	2.293	7.6	344.5	103.9		
Digilanid *B*	*F*	2.358	9.9	448.7	106.9	387.8 ± 27.68	98.6 ± 6.65
	F	2.196	9.3	401.5	94.8		
	M	2.265	8.1	376.2	105.2		
	F	2.663	10.2	250.1	65.3		
	F	2.110	8.6	484.4	118.8		
	F	2.343	9.0	358.5	93.3		
	F	2.343	8.5	430.2	118.6		
	M	2.442	10.1	403.8	97.6		

(Table II continued on p. 238)

Compound	Sex of Cat	Body Weight, Kg.	Heart Weight, Gm.	Fatal Dose by Body Weight, μg./Kg.	Fatal Dose by Heart Weight, μg./Gm.	Mean Lethal Dose by Body Weight, μg./Kg.	Mean Lethal Dose by Heart Weight, μg./Gm.
Digilanid *C*	*F*	2.597	10.8	219.1	52.7	232.6 ± 18.10	61.6 ± 3.50
	M	2.593	9.4	207.1	57.1		
	F	2.708	11.0	272.2	67.0		
	F	2.776	8.6	180.1	58.1		
	F	1.918	8.4	260.7	59.5		
	F	2.150	9.5	294.7	66.7		
	F	2.235	7.8	296.6	85.0		
	F	2.005	6.5	169.1	52.2		
Digoxin	*M*	2.626	9.6	181.2	49.6	235.0 ± 13.96	58.8 ± 3.61
	F	1.843	7.3	309.3	78.1		
	F	1.973	8.7	204.8	46.4		
	M	2.440	9.8	209.8	52.2		
	F	2.723	11.7	266.8	62.1		
	F	2.200	9.1	253.6	61.3		
	F	2.725	10.8	230.5	58.1		
	M	2.238	8.0	247.5	69.2		
Deacetyldigilanid *A*	*F*	1.779	8.3	530.6	113.7	469.2 ± 13.24	123.9 ± 3.49
	M	2.349	8.4	485.3	135.7		
	F	2.612	9.1	419.9	120.5		
	M	1.989	7.4	467.6	125.7		
	M	2.346	7.6	447.6	138.2		
	M	2.303	8.9	419.5	108.5		
	F	2.443	10.3	502.7	119.2		
	F	2.707	9.8	425.6	117.6		
	F	2.332	9.4	488.9	121.3		
	F	2.308	8.4	521.7	143.3		
Deacetyldigilanid *B*	*M*	2.522	9.8	420.3	108.2	548.4 ± 21.46	140.1 ± 6.19
	F	1.868	7.8	636.0	152.3		
	F	2.074	8.0	617.2	160.0		
	F	2.682	8.9	498.1	150.1		
	F	2.049	8.3	581.7	143.6		
	M	2.254	8.4	587.4	157.6		
	F	2.160	8.8	575.0	141.1		
	F	1.968	8.1	558.9	135.8		
	F	1.586	5.7	552.3	153.7		
	M	2.968	13.3	493.3	110.1		

33). It was found that for intravenous injection in cats at the rate of 1 cc. per minute, a solution of 1:100,000 was best suited for digoxin and digilanid *C;* one of 1:50,000, for digilanids *A* and *B;* and one of 1:25,000, for deacetyldigilanids *A* and *B*. Upon the death of each animal, the heart was dissected out and weighed, so that the individual dose could be calculated on the basis of heart weight, in addition to body weight.

RESULTS

The results in cats are shown in Table II. There is a high correlation between heart weight and body weight. It should be noted that digilanids *A* and *B* are less potent in cats than digilanid *C*. This is in agreement with our predecessors' reports (see Table I). Digoxin and digilanid *C* have practically the same activity. In cats, the larger molecule of the latter apparently does not diminish the potency as compared with the former, namely, digoxin. Previously, it was demonstrated that periplocin was actually stronger than periplocymarin while K-strophanthoside proved weaker than K-strophanthin-β (34). Thus, the effect of the extra sugar molecule on the activity of simpler glycosides is unpredictable. Aglycones are, however, less potent than their parent glycosides (35)—indicating the importance of the sugar radicals in simpler molecules.

According to our data, in cats (Table II) deacetyl-

TABLE III.—ASSAY IN FROGS BY THE 1-HR. METHOD

Compound	Dose, μg./Gm.	No. of Frogs in Systole/ No. of Frogs Used	SD_{50} ± S.E., μg./Gm.
Digilanid *A*	1.3	2/10	1.61 ± 0.13
	1.6	5/10	
	2.0	4/5	
	3.0	5/5	
Digilanid *B*	5.0	0/5	5.94 ± 0.60
	6.0	3/5	
	7.0	4/5	
	9.0	5/10	
Digilanid *C*	2.75	0/5	6.24 ± 0.44
	3.5	1/5	
	6.0	4/10	
	7.0	8/15	
	8.0	12/15	
	9.0	5/5	
Digoxin	3.0	0/5	4.22 ± 0.32
	4.0	6/10	
	5.0	6/10	
	6.0	5/5	
Deacetyl-digilanid *A*	1.6	2/10	2.40 ± 0.17
	2.0	3/10	
	2.5	6/10	
	3.5	7/10	
	4.0	5/5	
Deacetyl-digilanid *B*	5.0	1/5	5.58 ± 0.53
	5.5	2/5	
	6.0	4/5	
	7.0	4/5	

digilanids *A* and *B* are less potent than digilanids *A* and *B*, suggesting the favorable influence of the acetyl group on the cardiac action. The acetyl group in both compounds is presumably also attached to one of the digitoxose molecules as in the case of digilanid *C* (14). The differences observed in this investigation are far more decisive than those recorded by Rothlin (12, 15, 16). It must be pointed out that among the synthetic glycosides of strophanthidin the deacetyl members are much more potent than the acetyl derivatives (36).

The results in frogs as summarized in Table III are extraordinary in that they do not correspond to those in cats. In fact they reverse the order of activity in several instances. Our confidence in the data is enhanced by the fact that frogs from the same lot were employed for a repetition of the assay by the same person with similar results. Digilanid *A* is far more potent than digilanids *B* and *C*. Digoxin is significantly more active than digilanid *C*. While deacetyldigilanid *A* is less potent than digilanid *A*, deacetyldigilanid *B* and digilanid *B* have closely similar median systolic doses. The rating of activity of the six glycosides in frogs can be expressed as follows: digilanid *A*, 100; deacetyldigilanid *A*, 67.2; digoxin, 38.2; deacetyldigilanid *B*, 28.9; digilanid *B*, 27.1; and digilanid *C*, 25.8.

SUMMARY

Six glycosides of *Digitalis lanata*, digoxin, digilanids *A*, *B* and *C* and deacetyldigilanids *A* and *B*, have been assayed in cats and frogs. In cats, the order of activity from high to low is: digoxin and digilanid *C*, digilanids *A* and *B*, deacetyldigilanid *A*, deacetyldigilanid *B*. The results in frogs do not follow those in cats. The order of potency from high to low is: digilanid *A*, deacetyldigilanid *A*, digoxin, deacetyldigilanid *B*, digilanids *B* and *C*. The differences of the last three compounds are apparently not significant.

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The Effects of Life Cycle Therapeutic Dosage Administration of Drugs to Albino Rats

II. On Activity, Maze Learning and Relearning*†

By Lloyd L. Boughton

An earlier paper (1) has presented some of the effects of life cycle, oral therapeutic dosage administration of thirteen drugs to albino rats of the Wistar strain. Housing conditions, general care and diet, drug administration, formation of control and test groups were also discussed.

In the present study a maze was used primarily for the purpose of determining the effect of certain drugs administered daily for long periods of time on the activity of treated animals as compared to that of litter mate controls. Shirley (2) has found the revolving cage to be an excellent tool for studying the activity of the normal rat, but the writer has found it extremely inaccurate as a means of determining activity variations. The elevated *T*-maze has been found to express variations in activity drive with a high degree of accuracy. The animal is placed in a position which approaches the normal. He may run or not as he wishes.

A very simple pattern of elevated *T*-maze was used because of space limitations (Fig. 1). It was constructed of seventeen sections presenting sixteen culs-de-sac. The running strips were one inch wide and the total direct distance between starting point and goal was 35 ft.

Manually controlled drops were placed at seven points on the maze, the locations of which are shown in Fig. 1. These drops were used to prevent backtracking, a factor that must be eliminated if travel time and total time on the maze are to have any significance. The drops were constructed of light tin plates which were cushioned with rubber to insure a minimum of noise. They were operated individually by strings.

The same maze procedure was followed for all test and control groups run on the maze during the study. Experiments were started at 8 p. m., an animal from a control or test group being placed at the starting point at this time. Thereafter, the animals in the control and test groups were alternated on the maze until all had completed a run during the same evening or night. The animals were not touched after being placed on the maze. Food consisting of powdered crackers and milk was placed at the end of the maze, but the animals were not aware of its presence there until they had completed the first run. Similar dishes of food were placed at the right and left of the center of the maze in an attempt to rule out the sense of smell as a factor in maze learning.

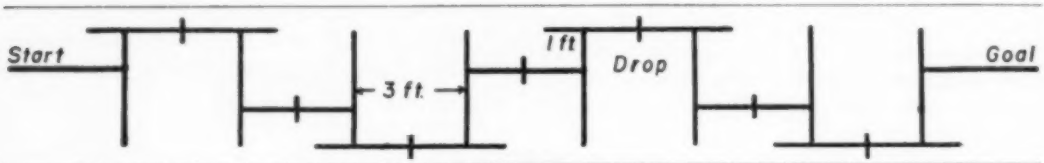

Fig. 1.—Diagram of *T*-Maze.

The maze was cleaned thoroughly each night after a group had completed a run, with a pinoleum antiseptic. Sections to which drops were not attached were reversed at this time.

Test groups to be run on the maze were deprived of the regular evening drug feeding which was given to the animals after the runs were completed. Purina Fox Chow briquets *ad libitum* were substituted for the normal cooked diet during maze learning and relearning periods. This change served to establish enough hunger to insure reasonable activity in most instances.

The animals were placed on the maze on successive nights until each rat in the test and control groups had completed three consecutive trials without error. A record of trials, errors, travel time, *i. e.*, time in motion, and total time on the maze was kept for each animal.

The drugs investigated in this manner are listed in Table I together with the dosage schedule. Summaries of results obtained, except for barbital and amytal, are presented in Tables II to VI, inclusive. The values listed are average values in each instance and represent the average for the group in completing three successive runs without error. The age of all groups, the number of rats per group and the length of drug feeding periods are included in the tables.

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Relearning trials were executed in the same manner as the learning trials, with the exception that the caffeine animals and their controls were given only six trials during relearning. The rats in these groups were considerably younger than those in other groups, and the interval between learning and relearning was only about half as long.

TABLE I.—DRUGS AND DRUG DOSAGE PER KG. OF RAT

Drug	70–200 Days, Mg.	200–300 Days, Mg.	300 Days to End of Period, Mg.	Average Human Dose, Gm.
Caffeine	3.0	6.0	9.0	0.20
Aspirin	4.3	8.6	12.9	0.30
Aminopyrine	4.3	8.6	12.9	0.30
Antipyrine	4.3	8.6	12.9	0.30
Barbital (Na)	7.1	14.2	14.2	0.50
Amytal (Na)	2.0	4.0	6.0	0.15
Phenobarbital (Na)	0.9	1.8	2.7	0.03
Alurate (Na)	2.0	4.0	6.0	0.15
Allonal				
Alurate	1.7	3.4	5.1	0.12
Aminopyrine	3.0	6.0	9.0	0.20

CAFFEINE (TABLE II)

Cheney (3) has studied the effect of caffeine on reaction time in humans. Doses below 3.0 mg./Kg. body weight had no effect, while doses between 3.0 and 4.0 mg./Kg. produced variable effects. However, doses above 5.0 mg./Kg. always produced a decrease in reaction time over at least a 3-hr. period.

Horst and Jenkins (4) obtained similar results with doses as low as 2.0 mg./Kg., the effect frequently being observed for 24 hrs. after administration. The doses used by these experimenters for humans compare favorably with the doses used by the writer for albino rats.

Lashley (5) has given caffeine to albino rats 10 min. before placing the animals on the maze, in doses corresponding by weight to 4- and 8-grain doses for man. He states that caffeine retards learning in rats in direct proportion to the size of the dose, and that large doses of the drug result in increased activity and reduced accuracy of performance.

Macht (6) has given caffeine to trained adult rats in doses varying from 10 to 50 mg./Kg. Excitement was produced in 71% of the experiments, while depression resulted in 24%. No effect was observed in 5%.

From the results presented in Table II, it would seem that caffeine as administered during this study stimulated all phases of maze behavior in female rats. A marked increase in activity was especially noticeable while these animals were on the maze. The normal animal, or an animal not motivated by some type of drive such as thirst or hunger, will frequently loiter at the starting position for long periods, and will usually feel his way rather carefully as he progresses. The caffeine females, however, started forward almost at once and actually appeared to be running at full speed even before the maze was learned. They would frequently run into a cul-de-sac so rapidly that they would be unable to stop. Although they would often run over the end, they were invariably able to pull themselves back to the top of the running strip. This excessive activity was not observed in any other group placed on the maze.

As has been stated in an earlier paper (1), the caffeine females evidenced an increased activity in the cage. They also appeared to have increased appetites, although there was no evidence of an increase in food consumption. The caffeine females were also considerably below their controls in weight at this period. This weight and apparent appetite difference may have been responsible, in part at least, for the marked increase in activity in the caffeine females. A lack of hunger drive was evidenced, however, by the fact that the animals infrequently ate the food when finally reaching goal.

Relearning trials for females produced about the same activity and error variations as did learning.

Horst and Willson (7) have reported a sex variation in humans in the effects of caffeine, the dosage ratio for men and women being about 2:1.

The results presented in Table II suggest a similar sex variation for albino rats, for, while caffeine stimulated all phases of maze behavior in females, this effect is very slight or completely lacking for males. The per cent of errors is observed to have been increased for caffeine males during both learning and relearning. During the six trials of relearning the six caffeine males made, respectively, five, seven, eight, two, four and three errors while the

TABLE II.—MAZE LEARNING AND RELEARNING OF CAFFEINE GROUPS, MALE AND FEMALE

	Males[a]			Females[b]		
	Test Group, 6	Controls, 5	Variation from Controls, %	Test Group, 7	Controls, 5	Variation from Controls, %
LEARNING						
Av. trials	15	17	−13	18.4	24.4	−24
Av. errors	60	49	+22	55.0	78.0	−29
Travel time	9.7 min.	11 min.	−12	9.7 min.	15.4 min.	−37
Total time	48.0 min.	51 min.	− 6	29.3 min.	71.0 min.	−59
RELEARNING[c]						
Av. errors	4.8	1.8	+17	13.0	16.6	−22
Travel time	146 sec.	156 sec.	− 6	144 sec.	208 sec.	−30
Total time	10.2 min.	7.3 min.	+38	9.9 min.	16.7 min.	−40

[a] Initial age 38 weeks, 28 weeks on drug.
[b] Initial age 39 weeks, 29 weeks on drug.
[c] Six trials for all groups: males, 6 weeks after learning; females, 11⅓ weeks after learning.

five controls made one, five, one, one and one errors. The six caffeine males made sixteen perfect runs during relearning, an average of 2.7. The five controls made twenty-three perfect runs, an average of 4.6.

ANTIPYRETIC DRUGS

There is little evidence in the literature of antipyretic drug effects as a result of continued administration, upon activity or learning. Jones (8) has attempted to determine the effect of aspirin upon learning in human beings and states that the drug seems to have a neutral effect.

Macht and Bloom (9) have studied the effect of single doses of quinine sulfate, salol, sodium salicylate, acetanilid, phenacetin, antipyrine and aminopyrine on the behavior of rats in the circular maze. Relatively small doses of 10 mg. or less were used, the performance of the animals being determined before, ½ hr. after and 3 hrs. after administration. They conclude that all of the antipyretics depress the behavior and memory habits of rats. Antipyrine and aminopyrine were the most effective in this respect, according to these authors.

The effects of three antipyretic drugs on maze activity and maze learning and relearning have been studied during this investigation. Two of these, antipyrine and aminopyrine, are included in the reference presented above. The third antipyretic, aspirin, is similar in its actions to the salicylates two of which were studied by Macht and Bloom (9).

Aspirin effects (Table III) on maze performance during learning are rather varied although the tendency is toward depression. Total time on the maze was increased significantly for both test groups, as was travel time for males. The relearning data for females reveal further evidence of depression, although a negative variation for total time has replaced a rather high positive variation obtained during learning. This change is even more marked for males, from +62% during learning to −16% during relearning. Trials and errors for males reveal no effect. The same data for females, while significantly higher than for the controls in three instances, show a slight decrease in average errors during learning.

Aminopyrine (Table IV) may be said to have had no depressant effect on maze behavior during learning. There is, in fact, a significant decrease in total time as compared to controls. Antipyrine, however, has caused some depression, especially in activity on the maze.

There can be little question as to the depressant effect of these two antipyretics during the relearning period. Large positive variations, coupled with the fact that the animals appeared completely lost during much of the relearning process, lead the writer to concur with Macht and Bloom (9) in their state-

TABLE III.—MAZE LEARNING AND RELEARNING OF ASPIRIN GROUPS, MALE AND FEMALE

	Males[a]			Females[b]		
	Test Group, 7	Controls, 6	Variation from Controls, %	Test Group, 6	Controls, 6	Variation from Controls, %
LEARNING						
Av. trials	21	19.7	+ 7	17	15	+13
Av. errors	106	105.0	+ 1	68	70	− 3
Travel time	16 min.	12.4 min.	+29	8.5 min.	8.1 min.	+ 5
Total time	118 min.	72.6 min.	+62	68 min.	54 min.	+26
RELEARNING[c]						
Av. trials	11.8	11.7	+ 1	10.0	8.7	+15
Av. errors	23.0	23.5	− 2	18.7	16.3	+15
Travel time	5.6 min.	4.0 min.	+40	5.3 min.	3.9 min.	+36
Total time	20.7 min.	24.4 min.	−16	17.6 min.	18.5 min.	− 5

[a] Initial age 70 weeks, 60 weeks on drug.
[b] Initial age 62 weeks, 52 weeks on drug.
[c] Males, 10½ weeks after learning; females, 12½ weeks after learning.

TABLE IV.—MAZE LEARNING AND RELEARNING OF AMINOPYRINE AND ANTIPYRINE GROUPS, FEMALE

	Aminopyrine			Antipyrine	
	Test Group, 8	Variation from Controls, %	Controls, 7	Test Group, 7	Variation from Controls, %
LEARNING[a]					
Av. trials	17.3	+ 2	16.9	18.0	+ 6
Av. errors	79.0	− 1	79.4	86.4	+ 9
Travel time	7.5 min.	+ 3	7.3 min.	8.7 min.	+19
Total time	53.4 min.	−17	64.6 min.	74.3 min.	+15
RELEARNING[b]					
Av. trials	14.9	+26	11.8	16.0	+36
Av. errors	43.1	+75	24.6	55.0	+124
Travel time	7.3 min.	+30	5.6 min.	7.9 min.	+41
Total time	46.2 min.	+113	21.7 min.	43.0 min.	+98

[a] Initial age 59 weeks, 49 weeks on drug.
[b] Twelve weeks after learning.

ment that aminopyrine and antipyrine are powerful depressants when judged by their effects on maze behavior.

BARBITURIC ACID DERIVATIVES

Omwake (10) has given 100 mg./Kg. doses of barbital to male and female rats on alternate days for 4½ months, reporting only a slight decrease in activity. The ability to locate a reward in a maze in 30 min. showed no significant differences between treated and control animals, but the percentage of barbital rats successful in 5 min. was definitely lower, according to this author.

Fields (11) has found that the daily administration of ⅛-grain doses of phenobarbital to rats for 18 days did not interfere with learning or retention of maze patterns. The phenobarbital animals were more active but made more errors.

Russell and Hunter (12) have completely anesthetized rats with sodium amytal following five trials on the maze. Results obtained from five trials following anesthesia failed to show a significant difference between the retest records of normal animals and the retest records of anesthetized animals.

Williams (13), Williams and O'Brien (14) and Mendenhall (15) have determined the effects of sodium phenobarbital on maze performance and all report a marked inferiority in the drugged animals as measured by errors and time, in both learning and relearning. A similar inferiority was observed by Mendenhall (15) in two reasoning problems.

The maze behavior effects of five barbituric acid derivatives, including allonal, were studied during this investigation and the results bear out the findings of Williams (13), Williams and O'Brien (14) and Mendenhall (15).

Five barbital males were placed on the maze after 69 weeks of continuous drug feeding, at 79 weeks of age. None of the five animals showed more than a suggestion of activity during the first seven trials. One hour before the eighth trial was to begin, the group was given 5 mg. of caffeine per rat orally. When placed on the maze following this dose of caffeine, three of the five climbed down immediately, one left the maze at the eighth intersection, and the fifth animal completed the run after 10 min. and 16 sec., making sixteen errors. In ten trials for each of the five barbital animals, only four completed runs were made, two by each of two animals.

Five amytal males, litter mates of the barbital males, were continued on the maze for twelve trials with ten litter mate controls. The amytal animals were sluggish as compared to the controls but they were much more active than the barbital animals. During these twelve trials the amytal rats made 26% more errors than the controls, the travel time was 41% greater and the total time 44% greater. The

TABLE V.—MAZE LEARNING AND RELEARNING OF SODIUM PHENOBARBITAL GROUPS, MALE AND FEMALE

	Males[a]			Females[b]		
	Test Group, 6	Controls, 6	Variation from Controls, %	Test Group, 6	Controls, 6	Variation from Controls, %
LEARNING						
Av. trials	21	19.6	+ 8	19.4	15	+29
Av. errors	126	104.7	+20	93.5	70	+37
Travel time	15.4 min.	12.7 min.	+24	9.7 min.	8.1 min.	+20
Total time	93.7 min.	72.6 min.	+28	64.0 min.	54.0 min.	+19
RELEARNING[c]						
Av. trials	16	11.7	+37	13.0	8.7	+59
Av. errors	36	23.5	+53	32.0	16.3	+106
Travel time	6.7 min.	4.0 mm.	+67	6.0 min.	3.9 min.	+37
Total time	63.3 min.	24.4 min.	+155	65.4 min.	18.5 min.	+250

[a] Initial age 70 weeks, 60 weeks on drug.
[b] Initial age 62 weeks, 52 weeks on drug.
[c] Males, 10½ weeks after learning; females, 12½ weeks after learning.

TABLE VI.—MAZE LEARNING AND RELEARNING OF ALLONAL AND SODIUM ALURATE GROUPS, FEMALE

	Allonal			Alurate	
	Test Group, 8	Variation from Controls, %	Controls, 7	Test Group, 7	Variation from Controls, %
LEARNING[a]					
Av. trials	19.6	+13	17.3	18.7	+ 8
Av. errors	107.0	+32	81.0	97.0	+20
Travel time	11.0 min.	+30	8.5 min.	9.6 min.	+13
Total time	69.3 min.	+26	55.1 min.	58.8 min.	+ 6
RELEARNING[b]					
Av. trials	17.3	+66	10.4	14.9	+43
Av. errors	59.0	+124	26.2	51.2	+96
Travel time	9.2 min.	+84	5.0 min.	8.3 min.	+66
Total time	48.0 min.	+95	24.6 min.	42.0 min.	+70

[a] Initial age 50 weeks, 40 weeks on drug.
[b] Eleven weeks after learning.

ten controls made sixteen perfect runs during the period as compared to five for the five amytal rats. Three of the ten controls failed to make a perfect run in twelve trials as compared to three of five amytal rats.

Maze behavior effects of sodium phenobarbital on male and female rats, and of allonal and sodium alurate on females, are presented in Tables V and VI. Sluggishness was observed during learning in both phenobarbital groups and allonal. It was very evident for all test groups during the relearning period. Attention is called to the fact that the phenobarbital females required $3^1/_2$ times as much total time during relearning as did their litter mate controls, while the phenobarbital males required $2^3/_4$ times as much as their controls. Travel time for all groups shows a less marked but consistent positive variation for all test groups.

All test groups were inferior in learning to the controls and this inferiority was even more marked during relearning.

Allonal seems to have exerted a stronger depressant effect than alurate, the barbituric acid derivative contained therein. This is undoubtedly due to the presence of aminopyrine in the compound since this drug has been shown to exert a powerful depressant effect upon maze performance during the relearning period.

SUMMARY

The effects of continuous daily oral administration of nine commonly used drugs on maze performance in albino rats have been presented. The drugs were administered in doses approximating, on a weight basis, the average daily therapeutic dose for adult humans. Test groups and litter mate controls were subjected to maze learning after drug feeding periods varying with the group, from 28 to 69 weeks. All groups, with the exception of sodium barbital and sodium amytal, were allowed to relearn the maze after rest periods varying from 6 to $12^1/_2$ weeks.

Caffeine has stimulated all phases of maze performance in female rats during both learning and relearning. Activity was affected most noticeably. Caffeine males failed to show a similar stimulation. There were, in fact, some evidences of depression during relearning in these animals.

Of three antipyretic drugs investigated, aspirin had the least effect. There is, however, some evidence of depression during learning in males and during relearning in females.

Aminopyrine exerted no depressant effect during learning. The total time on the maze was significantly less than for controls. Antipyrine animals exhibited some depression during learning and both drug groups were markedly depressed in all phases of maze performance during relearning.

All five barbituric acid derivatives were found to exert a powerful depressant effect, more especially on activity as judged by travel time and total time records. While it is difficult to draw comparisons because of differences in age and length of feeding periods for the barbiturate groups, the author would list the five barbituric acid derivatives tested, in order of increasing depressant effect, as follows; sodium alurate, allonal, sodium phenobarbital, sodium amytal, sodium barbital.

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Sterility Tests on Chemical Preparations for Parenteral Use*

By Bernard L. Blumberg

In a report on the sterility of ampul preparations made by the Council on Pharmacy and Chemistry of the American Medical Association (1), injectable ampul and other preparations were classified into four general groups. Group I included products of a biological nature which are obtained from plant and animal tissues and are very susceptible to bacterial contamination; group II consisted of bactericidal substances such as soluble mercury and other heavy metal salts; group III included glucosides and preparations containing sugars or nonantiseptic carbon compounds in which bacteria and molds may grow; and group IV represented inorganic and organic substances in which the growth of bacteria is not likely, but which are not bactericidal and therefore may contain viable bacteria or spores. Chemical preparations containing preservative in bacteriostatic concentration, and vegetable oil suspensions of insoluble mercury, bismuth and arsenic compounds were also relegated to this group. This report noted further that some of the firms producing ampuls in these groups were testing their products for sterility by the National Institute of Health method, some by other methods, and some were making no tests for sterility.

The increasing necessity for sterility tests for routine control purposes is apparent from the growing number of medicaments marketed in ampuls, and from the researches of Davis (2) and others. Their work with experimentally infected solutions of substances for parenteral administration has shown that such substances may be classified as non- or slightly germicidal, moderately germicidal, and strongly germicidal. Moreover, certain medicinal solutions which are nongermicidal and not conducive to spore germination may contain viable organisms after a 3-day fractional sterilization at 80° C. for 1 hr.

Directions for official sterility tests on liquids and solids appeared in U. S. P. XI and its Supplements, and N. F. VI (3). Apparently the prototype for these directions is the National Institute of Health sterility testing method, which is a well-established procedure for biological products. However, this method seems unnecessarily time consuming as a routine procedure for chemical products, especially those in group IV.

With respect to chemical ampuls, this author believes that the official sterility test may be considerably modified. The modified procedure should facilitate routine testing without sacrificing in any way the ultimate value of the test.

The purpose of this paper is to record the modifications which have been investigated. One series of experiments was designed to determine whether a broth prepared from a dehydrated infusion broth (Difco heart infusion broth) could be substituted for the official fresh infusion broth, and also whether the Durham fermentation tube could be substituted for the Smith fermentation tube. The second series of experiments presents the results obtained with a large number of commercial preparations.[1] Duplicate samples of these preparations were tested by the author, using a Smith tube–U. S. P. broth, and by Mr. J. W. Glaser, New York, N. Y., using a Durham tube–Difco broth. Practically identical methods of sampling and inoculating were used by both investigators. The two laboratories in which the tests were conducted were quite different in construction, and were some three or four miles apart.

EXPERIMENTAL

Cultures.—Staph. aureus, E. coli, B. subtilis and *Cl. novyi* were used for the study of comparative growth in Smith tube–U. S. P. broth, Smith tube–Difco broth, Durham tube–U. S. P. broth, and Durham tube–Difco broth. All cultures except

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[1] The author is pleased to acknowledge the cooperation of Premo Pharmaceutical Laboratories, New York, N. Y.

Cl. novyi had been carried along as stock cultures for at least eighteen months, and had been checked occasionally for identity and purity. *Cl. novyi* had been obtained about six months previously from the A. T. C. C. and had been carried in pure culture. Stock cultures were maintained on beef extract broth agar (3 Gm. of beef extract, 5 Gm. of peptone, 5 Gm. of NaCl, 15 Gm. of agar and 1000 ml. of water; *p*H about 6.8) slants, except *Cl. novyi*, which was cultured in B. B. L. thioglycollate medium. Stock cultures which were used to inoculate initial subcultures were four to six weeks old. The initial subcultures were prepared by transferring a small quantity of growth from the respective stock cultures to tubes containing 10 cc. of beef extract broth (10 cc. of thioglycollate medium for *Cl. novyi*). After 24 hrs. incubation at 37° C., each subculture was again subcultured in 10 cc. of liquid medium as before, the transplant being made with a 4-mm. loop bent at an angle. This subculturing was repeated through three successive 24-hr., 37° periods, and the fourth subculture was then diluted after it had been incubated 12, 18, or 24 hrs. at 37° C., as noted in Table I. The macroscopic appearance of the liquid culture and a gram-stained smear served as checks on the culture before dilution.

Dilutions.—Dilutions were effected with sterile serological pipettes and sterile 0.8% saline solution contained in glass-stoppered flint glass dilution bottles. The volume of inoculum and dilution finally employed for each group of experiments are indicated in Table I. Preliminary experiments revealed that 10^{-5} dilutions (this is the dilution stated in the medium sensitivity test of the U. S. P. XI, Second Supplement (4) for *E. coli* and *Cl. novyi*) would produce growth in 100% of the tube–medium combinations noted above. Higher dilutions were necessary, therefore, in order to determine what number of positives any particular tube–medium combination would give, when all were inoculated under comparable conditions. An approximation of the number of organisms per cc. of the inoculating dilution was obtained in some cases by plate count. Three 1-cc. quantities of this dilution were plated on U. S. P. broth containing 1.5% agar, and three 1-cc. quantities on Difco broth containing 1.5% agar. Six plates were thus prepared for each organism except *Cl. novyi*. A preliminary attempt to plate the latter and incubate the plates anaerobically indicated that the procedure would seriously interfere with the rest of the inoculation time schedule.

Tubes and Inoculation.—The Smith tubes employed in these experiments were of the S. A. B. type and 35-cc. capacity. The Durham tubes consisted of a 6 × 1-in. outer tube and a 3 × $^3/_8$-in. inverted inner tube, and contained 25 cc. of medium. Smith tubes were never covered with an anaerobic seal. Within ten minutes after inoculation, about 50% of all Durham tubes used were covered aseptically with a 1-in. layer of sterile high-viscosity mineral oil (Sonneborn Kaydol), or yellow petrolatum U. S. P. (see Table I). All tubes were glass capped. In every series of tubes inoculated, all four tube–medium combinations were represented. Inoculation of each four tubes in a

TABLE I

Culture	Age of Culture in Hrs. when Diluted	Incubation Time, Hrs.	Vol. of Inoculum, Cc.	Dilution	No. of Organisms in 6 Cc. of Dilution as Detd. by Plating	Smith Tubes–Difco Broth		Smith Tubes–U. S. P. Broth		Durham Tubes–Difco Broth				Durham Tubes–U. S. P. Broth			
						No. of Tubes Seeded	No. of Tubes Positive	No. of Tubes Seeded	No. of Tubes Positive	No. of Tubes Seeded		No. of Tubes Positive		No. of Tubes Seeded		No. of Tubes Positive	
										With Oil	Without Oil	With Oil	Without Oil	With Oil	Without Oil	With Oil	Without Oil
Cl. novyi	12	60[c]	1.0	10^{-6}	ND[a]	3	1	3	2	2	2	0	1	2	2	0	0
	12	60[c]	0.1	10^{-6}	ND	2	0	2	0	2	2	0	0	1	2	0	0
	18	48[c]	1.0	10^{-6}	ND	3	3	3	3	3	3	1	1	3	3	3	3
	24	116[d]	1.0	10^{-7}	ND	6	6	6	3	3[b]	3	3	3	3[b]	3	1	3
B. subtilis	12	36[c]	1.0	10^{-5}	ND	2	0	2	1	1	2	0	1	1	2	1	2
	12	36[c]	0.1	10^{-5}	ND	1	0	1	0	1	2	0	0	1	2	0	0
	12	48[c]	1.0	10^{-6}	1	3	2	3	0	3	3	1	1	3	3	0	1
	24	90[d]	1.0	10^{-7}	1	6	6	6	2	3[b]	3	0	2	3[b]	3	0	2
E. coli	12	36[c]	1.0	10^{-6}	ND	2	2	2	2	1	2	1	2	1	2	1	2
	12	36[c]	0.1	10^{-6}	ND	1	0	1	1	1	2	1	2	1	2	1	2
	12	48[c]	1.0	10^{-10}	1	3	0	3	0	3	3	0	0	3	3	2	0
	24	90[d]	1.0	10^{-9}	7	6	3	6	3	3[b]	3	2	2	3[b]	3	2	3
Staph. aureus	12	36[c]	1.0	10^{-6}	ND	2	2	2	2	1	2	1	2	1	2	1	2
	12	36[c]	0.1	10^{-6}	ND	1	0	1	0	1	2	1	1	1	2	0	1
	12	48[c]	1.0	10^{-9}	3	3	2	3	0	3	3	1	0	3	3	1	2
	24	90[d]	1.0	10^{-8}	123	6	6	6	6	3[b]	3	3	3	3[b]	3	3	3

[a] Not determined. [b] Solid Petrolatum. [c] Incubation temperature, 37° C. [d] Incubation temperature, 32° C.

series was carried out by delivering four 1-cc. quantities of dilution from a 5-cc. serological pipette; the order of pipetting was planned to minimize the variation in concentration of inoculum which might exist at different levels of the pipette. When the final dilution had been obtained, the time required to inoculate any comparative series was not more than fifteen minutes.

Media.—U. S. P. broth was prepared in a manner similar to the alternative method outlined in U. S. P. XI, Second Supplement (3). For each batch of broth, fresh beef hearts were purchased from local butcher shops. Difco broth was prepared according to directions stated on the label of the container. The concentration of dextrose in all media was 0.04%. Difco medium was always made fresh on the day required; U. S. P. broth was made every one to four weeks, and was sterilized in bulk (not more than 700–800 cc. to each container) at 15 lbs. for 20 min. All tubes of broth were inoculated shortly after they had cooled to room temperature. Sterility of medium was controlled in each experiment by leaving about 10% of the total number of tubes uninoculated. Approximately one-half of these control tubes were covered with oil or vaseline seal to check on the sterility of the sealing material and the method by which it was added. Occasional checks on both media prior to inoculation indicated that the *p*H was regularly 7.4–7.6. *p*H determinations were made with a Hellige glass standard comparator and bromothymol blue indicator.

Observations.—Observations were made over a 7-day period. The time of incubation in Table I represents the number of hours beyond which no more positives appeared. Tubes were regarded as positive when growth was characteristic macroscopically; some typical growth, positive tubes in every series were checked by gram-stained smears. Occasional tubes which did not appear typical were always smeared and confirmed microscopically. Tubes in which there was no macroscopic evidence of growth after seven days were considered negative. All results are tabulated in Table I.

DISCUSSION AND CONCLUSIONS

For determining the suitability of medium for sterility testing, the U. S. P. XI, Second Supplement (4), directs the use of 18–24-hr. broth cultures of *Cl. novyi* and *E. coli* diluted 10^{-6}. If the medium is satisfactory, 1-cc. inocula of these dilutions should grow. With this criterion as a basis, experiments were performed with cultures of *Staph. aureus, E. coli, B. subtilis and Cl. novyi.* The tube–medium combinations selected were Smith–U. S. P., Smith–Difco, Durham–U. S. P. and Durham–Difco. Preliminary tests indicated that all four combinations manifested growth in 100% of the tubes inoculated with 1 cc. of 10^{-6} dilution, so more extreme dilutions were employed. Experiments utilizing approximately 300 tubes were performed; the results on 247 of these are given in Table I. Although any one tube–medium combination shows a somewhat higher or lower number of positive growths than another (see Table I), it seems reasonable to assume that the random distribution of the organism per cc. of inoculum may have been as contributory to the final figures as the sensitivity of the tube–medium. Final conclusions on this point would depend upon a series of experiments which could be statistically interpreted.

From the point of view of routine testing, some of the obvious advantages of a Durham tube–dehydrated broth setup are: The Durham tube is much less expensive, it can be cleaned and handled more conveniently, it does not require expensive special racks, and it occupies less room in the incubator; the dehydrated medium is readily available, it can be prepared fresh and quickly when required, and it probably gives a broth of more standard composition than the fresh infusion. The following disadvantages of such a setup have been suggested: lack of ability of the Durham tube to support the growth of microaerophils and anaerobes without special precautions, inability of dehydrated medium to support the growth of bacterial contaminants in chemical ampuls and expensiveness of dehydrated medium.

There are counterconsiderations for each of these points. The experiments reported here indicate that the Durham tube–Difco medium will, without special precautions, support the growth of organisms representative of possible contaminants in chemical ampuls to a degree comparable to the Smith tube–U. S. P. broth. The material expense of dehydrated medium is more than that of the fresh infusion. However, the time saved in preparation, aside from other advantages previously noted, largely offsets this expense. This factor seems especially important in laboratories using not more than one to two liters of medium weekly.

When ampul products are biological in nature, one may assume that contamination with anaerobes or fastidious pathogens is not improbable. Therefore a tube–medium combination which would provide optimum conditions for these organisms is highly desirable. On the other hand, this author has not found any references, with perhaps the exception of Gershenfeld's (5), indicating that such organisms are present in chemicals or chemical ampuls. If they do occur, it appears questionable whether the official tube–medium would enhance the possibilities of detecting them. A brief survey of the abstract literature for contaminants in stock chemicals and chemical ampuls reveals that the organisms which have been isolated (6, 7, 8) are saprophytic cocci and rods. One investigation (8) of 133 samples, of which 44 were found to contain bacteria, states that anaerobic and pathogenic bacteria, tetanus bacillus and hemolytic streptococcus were absent. The present writer has isolated several cultures from contaminated commercial samples. All of these manifested copious growth when isolated and subcultured aerobically on ordinary beef extract liquid and solid media.

As previously noted, studies have been made to compare the results obtained on commercial samples when duplicates were tested by the official tube–medium and the Durham–Difco setup. These studies, covering more than 80 various samples to date, show that the latter compares favorably with the former. These experiments will be submitted for publication in the near future.

SUMMARY

A published classification of injectable substances into one biological and three chemical groups has been reviewed and considered in relation to the present official sterility test for liquids.

The equivalence of a tube–medium setup more convenient for routine sterility testing than the official tube–medium has been discussed, and the experiments relative thereto have been detailed.

For sterility tests on certain types of chemical ampuls, the experiments reported here, together with information published by other investigators, suggest that the use of a Durham tube–dehydrated medium may be satisfactory. In view of the many variables to be considered in experiments of this type, cooperative research on this problem would be desirable.

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Digitalis purpurea Naturalized in Newfoundland

*By M. L. Fernald**

It may be of importance to know that in portions of southern Newfoundland *Digitalis purpurea* is so thoroughly naturalized as to be an aggressive weed of roadsides, sandy embankments and shores. In 1910, the late Professor Karl M. Wiegand and I found it in great profusion near the mouth of Spruce Brook, on the shores of St. George Pond, slightly north of Stephenville Crossing (Spruce Brook Camps are well known to sportsmen). Our label of August 19, 1910, reads: "Escaped to banks of streams and lake (said to have spread for 5 miles around Spruce Brook)." An herbarium specimen collected by Dr. Perley Spalding in 1926 shows that it was still about Spruce Brook. In southeastern Newfoundland it is also becoming abundant. A label accompanying a specimen received from Mrs. Agnes M. Ayer in 1928 reads: "Established over several miles about Salmonier." It is evident that the cool and relatively moist summer climate of southern Newfoundland is favorable to the plant. Possibly this is a region which would supply a considerable bulk of digitalis already growing and where its cultivation would meet with immediate success. I suggest investigation of the present supply about Spruce Brook, where it is conspicuously abundant.

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The Hydrogen Ion Concentration of Solutions of Sodium Citrate Used for Preservation of Fluid Blood*

Edward C. Watts†

Cotter and MacNeal (1) in June, 1938, stated that "solutions of sodium citrate have been used to prevent coagulation of blood for many years, and these solutions have become increasingly important in preserving the fluid blood for transfer from one human being to another." The conclusion of this statement was, no doubt, at least partly inspired by the establishment of "blood banks" in many of the large medical centers of our country. Such a unit was opened for use at University Hospital, Ann Arbor, Michigan, early in 1940. Prior to this time the Hospital Pharmacy had been advised that solutions of sodium citrate would be used in increased quantities and preparation had been made, therefore, to anticipate the demands of the new department.

In their study of ampul solutions of sodium citrate intended for use as anticoagulants, Cotter and MacNeal found the solutions which they examined to be definitely alkaline, with *p*H values ranging from 8.0 to 8.5. They themselves prepared a 2.5% solution of sodium citrate in 0.6% sodium chloride, and found this solution to have a *p*H of 8.7. On the basis of their results they advised the use of citric acid to reduce the *p*H of solutions of sodium citrate intended for use as anticoagulants. Attempts to follow their directions in the laboratory of the Hospital Pharmacy were not satisfactory because the solutions so prepared were too acid. Consequently the amount of citric acid was reduced materially before the following satisfactory formula was obtained:

Sodium citrate U. S. P.	140.0 Gm.
Citric acid U. S. P.	0.5 Gm.
Distilled water, fresh, *q. s. ad.*	4.0 L.

Solutions made in accordance with this formula in pyrex glassware had a *p*H of 7.0 to 7.1. When filtered through paper, bottled in pyrex bottles and sterilized for one-half hour at 240° F. they underwent no change in *p*H value. Hundreds of bottles of this solution had been made and used with complete satisfaction when those in charge of the "blood bank" requested that the *p*H value of the finished citrate solutions be adjusted closer to the *p*H value of blood, namely, 7.3 to 7.4.

With this request came the realization, that inasmuch as the solution as formulated had a *p*H of 7.0 to 7.1 it would, in all probability, be necessary to eliminate the citric acid entirely in order to achieve the desired result. If this were so, there seemed to be some question as to the accuracy of Cotter and MacNeal's determination of the *p*H of solutions of sodium citrate and of the necessity for the use of citric acid. It was decided, therefore, to check back on one of the solutions described in the original article. This, a 2.5% solution of sodium citrate in 0.6% sodium chloride, was described as having a *p*H of 8.7. As prepared in the Hospital Pharmacy in pyrex glassware, the same solution had a *p*H value of 6.8. A second solution of 3.5% sodium citrate in freshly distilled water, made in pyrex ware was found to have a *p*H value of 7.3. This solution, when filtered through paper, bottled in pyrex bottles, sterilized for one-half hour at 240° F. and cooled, was discovered to be of the same *p*H as when first prepared. A third solution made in the same manner as the second, but bottled in a soft glass bottle, and sterilized similarly had a *p*H value of 8.8 after sterilization. This solution was more alkaline than any of the ampuls observed by Cotter and MacNeal, but it was sterilized in a container made of glass which was not intended for ampul medications.

Thinking that the high *p*H values obtained by Cotter and MacNeal may have been due to characteristics of some particular brand of salt, it was decided to study sodium citrate solutions made from the salts of different manufacturers. As only one brand was available in the laboratory, requests were made to three manufacturers of chemicals for *p*H readings on 2.5% and 3.5% solutions of their salt. Results were as follows.

Source	Sample	Solution, %	*p*H
Manufacturer No. 1	1	3	7.95
	2	3	7.55
Manufacturer No. 2	1	2.5	7.61
	2	3.5	7.58
Manufacturer No. 3	1	2.5	7.4
	2	2.5	7.4

The salt used in the Hospital Pharmacy's tests and from which a 3.5% solution with a *p*H of 7.3 was made, was of the same brand as that of manufacturer No. 3.

In order to compare other figures of the original publication, it was decided to examine ampul solutions of sodium citrate for *p*H values as was done by Cotter and MacNeal. Four well-known pharmaceutical manufacturers generously supplied 50-cc. ampuls of their sodium citrate solutions. In the following table these solutions are described briefly and the results of the examination of these ampuls are noted.

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* Presented to the Subsection on Hospital Pharmacy, Detroit meeting of the A. PH. A., 1941.

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Source	Nature of Solution	pH
A	Simple aqueous, 2.5%	6.6
B	Simple aqueous, 2.5%	7.1
C	Simple aqueous, 2.5%	6.9
D	Aqueous buffered sodium phosphate, 2.5%	7.4

In their paper, Cotter and MacNeal state that each of four ampul solutions of sodium citrate which they observed had a *p*H value of 8.0 or over. As the identity of the manufacturers of the ampuls used by Cotter and MacNeal in their observations is unknown, it is impossible to decide on a definite reason for such divergent results. So far as the ampul solutions examined in the Hospital Pharmacy are concerned, the variations in *p*H values (from 6.6 to 7.4) can, on the basis of the available information, be explained only by assuming that varying grades of glass were used in the manufacture of the empty ampuls.

CONCLUSIONS

1. The *p*H of a 3.5% solution of Sodium Citrate U. S. P. in freshly distilled water is 7.3.

2. This value does not change during the process of sterilization when such solutions are contained in properly prepared pyrex bottles.[1]

3. A simple, aqueous 3.5% solution of Sodium Citrate U. S. P., in freshly distilled water, is satisfactory for use as an anticoagulant, since it is of the proper *p*H and can be prepared, bottled and sterilized without a change of *p*H.[2]

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[1] Such bottles in the proper size are now readily available.

[2] *p*H values determined experimentally by the author are electrometric readings.

Some Notes on Chalk Mixture*

By Louis W. Schleuse† and J. Richard Stockton‡

A chalk mixture was included in the first "Pharmacopœia of the United States," and each revision has retained a mixture of prepared chalk (Table I). Although several changes have been made, the present official product is little different from the original, and both possess two objectionable features: They are fermentable, and the acacia is not a satisfactory suspending agent.

The unstable character of the preparation and the "positively injurious" effect (1) of the sucrose prompted Reynolds (2) in 1870, Kennedy (3) in 1872 and Hommel (1) in 1911 to recommend replacement of the sucrose with glycerin. The Fifth Revision of the "Pharmacopœia" (Convention of 1870) made such a change (Table I), and much later the Tenth Revision (1920) replaced both sucrose and acacia with glycerin (Table I), but neither modification survived the revision in which it appeared.

Jones (4) in 1870 and Rother (5) in 1873 proposed the use of compound chalk powders from which chalk mixture could be prepared when wanted. The Sixth Revision of the "Pharmacopœia" (1880) adopted a method of preparing chalk mixture from compound chalk powder, a practice retained in each subsequent revision except the Tenth (Table I).

Fantus and Snow (6) in 1922 also objected that fermentable carbohydrates (sugar and acacia) were "illogical . . . as ingredients of a medicine employed in fermentative diarrhea." They considered a carbohydrate-free formula containing prepared chalk, saccharin, cinnamon water and water, but found that "when kept exposed to sunlight for quite some time, it developed an offensive odor reminding one somewhat of hydrogen sulfide or of illuminating gas, with progressive diminution and final loss of cinnamon flavor." They observed no decomposition when fennel, anise or peppermint flavors were substituted for cinnamon.

In addition to the ease with which it is fermented, acacia is also objectionable as a suspending agent for prepared chalk. When the official chalk mixture is allowed to stand for a few days, all of the chalk

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TABLE I.—PHARMACOPŒIAL CHALK MIXTURES

Pharmacopœia	Prepared Chalk	Compound Chalk Powder	Acacia	Sucrose	Glycerin	Diluent and Flavor	
1820	℥ iss	...	℥ ss	℥ i	...	Oil cinnamon	♏ x
						Water	℥ xx
1830 (New York)	℥ ij	...	℥ i	℥ i	...	Peppermint water	℥ viij
1830 (Philadelphia)	℥ ss	...	℥ ij	℥ ij	...	Cinnamon water	
						Water, *āā*	℥ iv
1840	℥ ss	...	℥ ij	℥ ij	...	Cinnamon water	
						Water, *āā*	℥ iv
1850	℥ ss	...	℥ ij	℥ ij	...	Cinnamon water	
						Water, *āā*	℥ iv
1860	℥ ss	...	℥ ij	℥ ij	...	Cinnamon water	
						Water, *āā*	℥ iv
1870	℥ ss	...	℥ ij	...	℥ ss	Cinnamon water	
						Water, *āā*	℥ iv
1880	...	20 parts	...	...	...	Cinnamon water	40 parts
						Water	40 parts
1890	...	200 Gm.	...	...	...	Cinnamon water	400 cc.
						Water, *q. s.*	1000 cc.
1900	...	20 Gm.	...	...	...	Cinnamon water	40 cc.
						Water, *q. s.*	100 cc.
1910	...	20 Gm.	...	...	...	Cinnamon water	40 cc.
						Water, *q. s.*	100 cc.
1920	6 Gm.	...	...	...	10 cc.	Cinnamon water	40 cc.
						Dist. water, *q. s.*	100 cc.
1930	...	20 Gm.	...	...	...	Cinnamon water	40 cc.
						Dist. water, *q. s.*	100 cc.

settles as a hard, compact layer very difficult to resuspend.

The excellent suspending qualities and the freedom from toxicity[1] of bentonite led us in the summer of 1940 to investigate the applications of this mineral as a suspending agent for insoluble materials in diarrhea mixtures. It was found to be especially suitable for preparations containing bismuth subcarbonate or prepared chalk, which suggested that bentonite might be preferable to acacia in the official chalk mixture.

Furthermore, bentonite is a domestic product and is much less expensive than imported acacia.

EXPERIMENTAL

Materials Used.—Two commercial bentonites (subsequently designated 1 and 2), prepared chalk, soluble saccharin (in stock solution, 10 mg./cc.), cinnamon water, peppermint water, distilled water and compound chalk powder.

Replacement of Acacia and Sucrose by Bentonite and Soluble Saccharin in Chalk Mixture.—A 6% magma of each bentonite was prepared with the aid of a mechanical agitator and allowed to stand over night. A series of ten chalk mixtures was made from each bentonite magma according to the following formula:

Prepared chalk	3.0 Gm.
Bentonite magma	Variable
Soluble saccharin	0.01 Gm.
Cinnamon water, enough to make	50.0 cc.

The individual preparations differed only in bentonite content, from 0.3% in the lowest to 3% in the highest. These chalk mixtures were compounded by triturating 3 Gm. of chalk with the bentonite magma, followed by the addition of 1 cc. of soluble saccharin solution and enough cinnamon water to make the volume 50 cc. A sample of chalk mixture U. S. P. XI and a suspension of 3 Gm. of prepared chalk in 50 cc. of cinnamon water were used for comparison.

All were shaken at the same time and allowed to settle, examinations and photographs being made at suitable intervals. The photographs made at 1 hr. and at 20 hrs. are shown in Fig. 1 and Fig. 2, respectively. These satisfactorily show that the rate of settling is dependent upon the amount of bentonite present. But the photographs fail to show the extent of settling in the U. S. P. XI chalk mixture, in which after 20 hrs. the chalk had most completely settled to the bottom leaving, however, a small amount of fine chalk in suspension, thereby forming a hazy supernatant layer, opaque in the photographs.

Comparison of the two series of mixtures shows little difference in suspending powers of the two bentonites. Although the preparations containing less than 0.9% of bentonite (*i. e.*, less than 7.5 cc. of 6% magma per 50 cc.) settled appreciably within an

[1] "Bentonite meets the requirements of the test for arsenic, U. S. P. XI, p. 436, and the test for heavy metals, U. S. P. XI, p. 447." Private communication from American Colloid Company, Chicago, Ill., September 3, 1940.

Fig. 1.—Photograph of Chalk Mixtures after One Hour (Preparation Containing Only Chalk and Cinnamon Water Indicated by Asterisk).

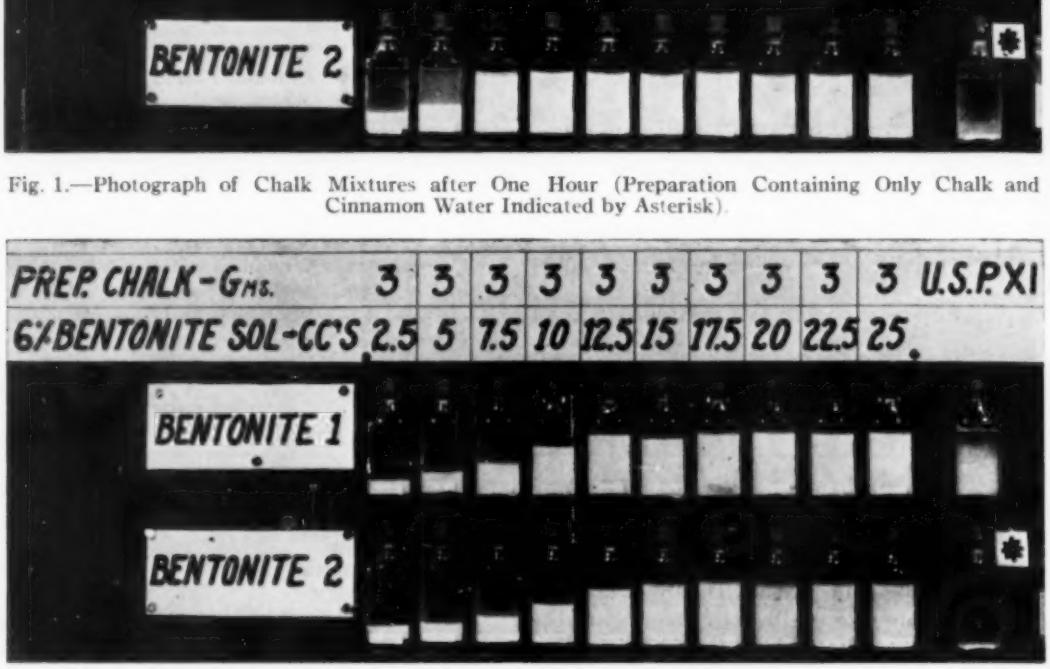

Fig. 2.—Photograph of Chalk Mixtures after Twenty Hours (Preparation Containing Only Chalk and Cinnamon Water Indicated by Asterisk).

hour, all resuspended easily on being shaken, even after standing for more than one year. Furthermore, in preparations containing bentonite in excess of about 2.4%, resuspension was slightly more difficult, though much less difficult than in the U. S. P. XI chalk mixture. For these reasons, a concentration of bentonite between about 0.9% and 2.4% seems most desirable.

The observation of Fantus and Snow on the production of a hydrogen sulfide odor in a mixture containing chalk, saccharin and cinnamon water was not confirmed, even after one year, although a toluene-like odor (perhaps the illuminating gas odor they described) was detected in all samples containing bentonite, especially in those samples containing larger proportions of bentonite magma. Two samples of this chalk mixture were set aside for more than a year, one in darkness and one exposed to sunlight; periodic examinations were made. The characteristic toluene-like odor developed in both within one to two months. Since this odor appears to depend upon the presence of cinnamon, the replacement of cinnamon by some other acceptable flavor seemed desirable.

Replacement of Cinnamon Water by Peppermint Water in Bentonite Formula.—A chalk mixture was prepared in which the cinnamon water in the above formula was replaced with peppermint water. The odor remained unchanged during the period of observation.

Suggested New Formula for Chalk Mixture.—On the basis of the foregoing results a chalk mixture containing 2% of bentonite was prepared as follows:

Prepared chalk	6.0 Gm.
Bentonite magma[2]	40.0 cc.
Soluble saccharin	0.02 Gm.
Peppermint water	40.0 cc.
Distilled water, enough to make	100.0 cc.

[2] Bentonite magma was prepared as follows:

Bentonite	50.0 Gm.
Distilled water, enough to make	1000.0 cc.

Sift the bentonite on the surface of 900 cc. of distilled water contained in a suitable vessel. When the bentonite has become thoroughly wetted, mix well by vigorous stirring or by means of a mechanical agitator. Allow to stand for at least 12 hrs.; strain through muslin, and add enough water through the strainer to make 1000 cc. Mix thoroughly.

Place the prepared chalk in a mortar and triturate until free from lumps. Add bentonite magma in small portions, triturating thoroughly after each addition until a uniform mixture results. Then add the peppermint water in which the soluble saccharin has been dissolved; transfer this to a graduated vessel and rinse the mortar with enough distilled water to make the product measure 100 cc. Mix thoroughly.

This product is entirely palatable and yields satisfactory preparations when combined in prescription form with elixir of phenobarbital, elixir of Nembutal, compound elixir of pepsin, elixir of pepsin and rennin, Liquid Takadiastase, Caripeptic Liquid, camphorated tincture of opium, milk of magnesia, milk of bismuth, aluminum hydroxide gel, bismuth subcarbonate, bismuth subgallate, bismuth subnitrate, Kaomagma, Kaopectate and tincture of belladonna. Addition of tincture of opium causes excessive thickening.

SUMMARY AND CONCLUSIONS

1. Chalk mixtures containing prepared chalk, soluble saccharin, cinnamon water, distilled water and variable amounts of bentonite were prepared. The bentonite seems superior to the more expensive acacia as a suspending agent for this purpose; concentrations between 0.9% and 2.4% are most suitable. Elimination of fermentable materials (sucrose and acacia) produced a biologically stable preparation, but some samples after long standing developed odors resembling toluene.

2. The toluene-like odor is not produced when cinnamon water is replaced by peppermint water.

3. An improved formula for chalk mixture containing prepared chalk, bentonite (magma), soluble saccharin, peppermint water and distilled water is suggested. This product is palatable and is compatible with many of the drug preparations that are prescribed with chalk mixture.

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The Toxicity of Benzoic Acid for White Rats*

By George P. Hager,† C. W. Chapman and E. B. Starkey

Benzoic acid is widely used in the preservation of food products (1) and pharmaceutical preparations (2), especially those with an acid reaction. Furthermore, because of its antiseptic action, it is used in ointments and dusting powders for the treatment of skin infections, in a 1% solution as a mouth wash (3), in a 0.5% solution for the treatment of chronic suppurating wounds (4) and in preparations for the treatment of tuberculosis, asthma and rheumatism (1).

In view of these many varied uses of benzoic acid, much interest attaches to its toxicity. It has been found that a rather broad margin of safety attends its use in these instances—0.5 Gm. daily produced no demonstrable effects in healthy persons and even 4 Gm. was not injurious (5, 6). Amounts far in excess of the quantities likely to be consumed with food are required to produce ill effects or cause the death of human beings or experimental animals. However, benzoic acid, one of the least harmful of food preservatives, should be avoided by persons with gastro-intestinal or renal diseases (7). Many attempts have been made to prepare compounds related to benzoic acid with even greater margins of safety in the preservation of food. For the purpose of comparing the effect of benzoic acid with that of certain of its substitution derivatives, a preliminary study of the acute toxicity of these compounds for white rats was undertaken.

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EXPERIMENTAL

A portion of the results of this work is the subject of this report. For the comparison of the value of the LD_{50} for white rats found in this investigation with the statements on the toxicity of this substance for human beings and various experimental animals reported in the literature, Table I was drawn up. These other results do not include a measure of the standard deviation and, therefore, the significance of the differences of the reported values cannot be calculated.

Table I.—Summary of the Investigations of the Acute Toxicity of Benzoic Acid

Subject	Dose	Effect
Dogs, cats, rabbits[a]	2 Gm./Kg.	Lethal dose
Rabbits (dry-fed)[a]	1.7 Gm./Kg.	Lethal dose
Rabbits (fasting)[a]	1.52 to 1.83 Gm./Kg.	Lethal dose
Guinea pigs (intraperitoneal)[a]	1.4 Gm./Kg.	Lethal dose
White rats (intravenous)	1.714 ± 0.037 Gm./Kg.	LD_{50}
Human[b]	1 to 1.5 Gm. (capsule)	Gastric pain, nausea and vomiting

[a] See reference (8).
[b] See reference (9).

The method of determining the acute LD_{50} reported here for white rats consisted in injection of an aqueous solution of sodium benzoate of appropriate concentration into the saphenous vein at a rate of 1 cc. per min. Five doses were employed in each test, the first of which was likely to kill none of the animals injected and the last of which was likely to kill all of the animals injected. The doses differed by a constant ratio, the logarithm of which was 0.07; and varied from 1.2 to 2.29 Gm./Kg. Each dose was administered to a group of five rats weighing from 90 to 150 Gm. The solutions employed were of such concentration (12% to 23%) that the dose, based on the weight of the rat, would be contained in about 1 cc. The solutions were prepared by dissolving the highly purified acid in a solution of an equivalent weight of sodium carbonate. Results were recorded hourly for 5 to 6 hrs. to ascertain rate of onset of action, symptoms, etc., and the LD_{50} calculated from the number of animals dead in 24 hrs.

The symptoms of benzoic acid poisoning observed during the course of these experiments were those resulting from a heightening of the reflexes of the central nervous system, and consisted of tremors, clonic and often tetanic convulsions, with death occurring during a remission. These symptoms appeared within a few minutes after the injection and progressed rapidly with deaths often occurring one-half to one hour after injection. Surviving animals generally showed none of the nervous manifestations, but salivation, vomiting and diarrhea were present. A marked diuresis was seen in some cases.

For the calculation of the LD_{50} from these experiments, the use of the maximum likelihood method for the statistical treatment of biological results would be very cumbersome and would likely fail to give values much different from those obtained by a less exacting method. Therefore, the LD_{50}'s were calculated by Kärber's method (10); and the standard deviation of the individual determinations was obtained after first fitting the observed mortalities to a straight line by a method developed by Irwin and Cheeseman (11). The weighted mean of the values thus obtained in three separate determinations was calculated. The standard error of this mean was then obtained in the usual manner. In a different set of experiments carried out in the same way and on the same days, the toxicity of a fluorine-substituted benzoic acid was studied. The results of these experiments are summarized in Table II.

TABLE II

Benzoic Acid	*p*-Fluorobenzoic Acid
LD_{50} ± *S. D.*, Gm./Kg. (P = 0.66)	
1.686 ± 0.105	1.982 ± 0.137
1.855 ± 0.076	1.687 ± 0.109
1.630 ± 0.062	1.434 ± 0.055
Weighted Mean (LD_{50} ± *S. E.*), Gm./Kg.	
1.714 ± 0.062	1.542 ± 0.107
(P = 0.66)	(P = 0.66)
1.714 ± 0.124	1.542 ± 0.214
(P = 0.95)	(P = 0.95)

In experiments of this type in which a small number of animals and a large number of doses are employed, it has been shown that the values obtained by the maximum likelihood method of calculation and by the method outlined above are very close (11).

By plotting the percentage mortalities observed in an individual determination, as ordinates, against the corresponding doses expressed as a logarithm of the ratio of the doses to the LD_{50} calculated from the experiment, the results of the three experiments can be combined in a single curve, the regression line. This can be conveniently done by plotting the percentage mortalities against the ratio on logarithmic probability paper (Fig. 1). Seventy-five animals were employed in the construction of this curve, and its steepness indicates a great uniformity in the response of white rats to benzoic acid.

The significance of the differences between the individual determinations and between the mean values was calculated by the formula

$$\frac{m_1 - m_2}{\sqrt{(S.\,E._1)^2 + (S.\,E._2)^2}}$$

where m is an individual LD_{50} or a weighted mean LD_{50} and $S.\,E.$ is the standard deviation of the corresponding individual LD_{50} or the standard error of the corresponding weighted mean LD_{50}. When the value of this quotient is 2 or greater, the difference between m_1 and m_2 is generally considered significant. That is to say that when the value is 2 or greater, the

Figure 1.—Regression Line for the Action of Benzoic Acid on White Rats.

observed difference could occur through chance or through an error in sampling only once in twenty times; and the chances are twenty to one that a greater toxicity is to be assigned to the substance whose LD_{50} is m_1 than to that whose LD_{50} is m_2. The application of this formula showed that no significant difference exists between the three values of the LD_{50} determined for benzoic acid. Likewise was the difference between the LD_{50} found for benzoic acid and that found for *p*-fluorobenzoic acid shown to be insignificant. On this basis, one is justified only in saying that benzoic acid and *p*-fluorobenzoic acid were found in these experiments to be of the same toxicity even though the absolute value of the weighted mean LD_{50} of benzoic acid is greater than that of *p*-fluorobenzoic acid. The use of statistical methods is therefore seen to be of great value in the study of the relation of chemical structure and physiological activity from a quantitative standpoint.

SUMMARY

The toxicity of benzoic acid when administered as the sodium salt intravenously to white rats corresponds closely to that reported for other animals. The value of the LD_{50} found in this investigation is 1.714 ± 0.124 Gm./Kg. ($P = 0.95$).

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